CORRECTED VERSION*

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 48/00, C12N 15/63, C07K 16/00

A1

(11) International Publication Number: **WO 00/06203**

(43) International Publication Date: 10 February 2000 (10.02.00)

(21) International Application Number: PCT/US99/16000

(22) International Filing Date: 15 July 1999 (15.07.99)

(30) Priority Data:
09/123,485 28 July 1998 (28.07.98) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: DNA CYTOKINE VACCINES AND USE OF SAME FOR PROTECTIVE IMMUNITY AGAINST MULTIPLE SCLEROSIS

(57) Abstract

A method for treating a mammal for inducing protective immunity against an autoimmune disease including the step of administering to the mammal a therapeutic composition including a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences. A method for treating a mammal for inducing protective immunity against an autoimmune disease including the steps of (a) removing cells of the mammal; (b) transducing the cells in vitro with a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences; and (c) reintroducing the transduced cells to the mammal. A pharmaceutical composition including (a) a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences; and (b) a pharmaceutically acceptable carrier. And an antibody raised against a cytokine expressed by cells transduced with a recombinant construct including an isolated nucleic acid sequence encoding the cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences.

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DNA CYTOKINE VACCINES AND USE OF SAME FOR PROTECTIVE IMMUNITY AGAINST MULTIPLE SCLEROSIS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to DNA vaccines and DNA vaccination, and more particularly, to DNA encoding cytokines and the use of same as DNA vaccines for inducing protective immunity against autoimmune diseases. Most particularly, the present invention relates to DNA encoding C-C chemokines and tumor necrosis factor alpha and the use of same for protective immunity against multiple sclerosis.

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) which, for many years and for a variety of experimental protocols, serves as a model for the human disease, multiple sclerosis (MS), a chronic degenerative disease marked by patchy destruction of the myelin that surrounds and insulates nerve fibers and mild to severe neural and muscular impairments, since in both diseases circulating leukocytes penetrate the blood brain barrier and damage myelin resulting in impaired nerve conduction and paralysis (1, 2).

Molecular biologic techniques were previously used to follow leukocyte trafficking to the site of inflammation at the CNS of EAE rats, and a model that characterizes this process as a sequential multi-step event was suggested (3).

At first, a very limited repertoire of T-cells, named "the primary influx" interact with their target antigen at the site of inflammation, leading to the activation of the blood brain barrier to express various adhesion molecules and thus to increase its permeability to circulating leukocytes (3, 4). Enhanced permeability of this barrier allows a non-selective influx of leukocytes, which are named "the secondary influx". This influx correlates with disease onset (3, 5). Subsequently, antigen specific autoimmune T-cells either become anergic or undergo programmed cell death (apoptosis) leading to a remission in disease severity (6). Inhibition of the secondary

influx, by either soluble peptide therapy or anti-adhesion molecule blockade effectively prevented, or even reversed, an ongoing disease even though the primary influx remained apparent at the site of inflammation (3-5, 7). Taken together these results not only suggest novel therapeutic strategies, but also emphasize the important role of the non-selective leukocyte influx to a site of inflammation.

Chemokines are chemoattractants that mediate leukocyte attraction and recruitment at the site of inflammation. As such, they are likely to be key mediators in the recruitment of the secondary influx of leukocytes at an inflamed target organ. This has motivated us to use the novel technology of naked DNA vaccination (8-17) and explore the therapeutic potential of anti-chemokine immunotherapy in EAE.

Based on the positions of the first two cysteines, the chemokines can be divided into four highly conserved but distinct supergene families C-C, C-X-C, C and the newly discovered C-X3-C (18, 19). The C-C family is primarily involved in the activation of endothelium and for chemoattraction of T cells and monocytes to the site of inflammation (20-32). The protective competence of anti-C-C chemokine based immunotherapy has been demonstrated by Karpus *at al.* who blocked EAE in mice by immunizing them with rabbit anti-mouse polyclonal antibodies against macrophage inflammatory protein-1α (MIP-1α) (33), and very recently by Gong *at al.* who used an antagonist of monocyte chemoattractant protein 1 (MCP-1) to inhibit arthritis in the MRL-lpr mouse model (34). In another study Berman *at al.* used in situ hybridization to demonstrate the dominant expression of MCP-1 in rat EAE brain (35).

In the course of reducing the present invention down to practice we have cloned each of the major C-C chemokines: MCP-1, MIP-1α, macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES) from EAE brains into an

eukaryotic expression vector and determined their capacity to block EAE when used as vaccines.

Thus, during the course of EAE various proinflammatory cytokines and chemokines are produced at the site of inflammation (40, 53-55). The pivotal role of one of these proinflammatory cytokines; tumor necrosis factor alpha (TNF-α), in EAE has been well characterized. TNF-α is produced by activated T cells (mostly Th1) and macrophages, and its elevated expression at the site of inflammation occurs during the critical phase of disease (55), at the time when the 'secondary influx' of leukocytes is apparent (3). Except for a single recent study carried out in genetically modified animals (56), all investigators agree that TNF-α contributes to the proinflammatory process in EAE and MS (57-71). Early studies have shown that IFN-γ and TNF-α together exhibit a synergistic effect on enhancing expression of adhesion molecules on endothelial cells (61), and on eliciting the inflammatory process, which can be reversed by either anti-adhesion molecule immunotherapy (4), or by blocking TNF-α (57-61). More recent studies have demonstrated that inhibition of TNF-α activity by either neutralizing antibodies, or soluble TNF receptor therapy, effectively prevent , or even reverse EAE (62, 64, 66-71). Overexpression of TNF-α at the CNS aggravated the disease (65), whereas genetically impaired expression of this gene inhibited disease development and progression (63).

A major disadvantage in treating chronic diseases with xenogenic neutralizing antibodies lies in their immunogenicity. This has motivated investigators to develop chimeric humanized antibodies (reviewed in 50), and monoclonal antibodies engineered with human Ig heavy and light chain yeast artificial chromosome (YAC) (51). However, following repeated immunization, these engineered antibodies do trigger an apparently allotypic response.

The therapeutic strategy of the present invention, is of advantage over the above methods since it resulted in the generation of immunity to autologous antigens.

There is thus a widely recognized need for, and it would be highly advantageous to have, methods and compositions enabling vaccination with DNA encoding cytokines, such as C-C chemokines and tumor necrosis factor alpha and the use of such vaccination for protective immunity against multiple sclerosis, devoid of the limitations associated with the use of neutralizing antibodies.

SUMMARY OF THE INVENTION

DNA vaccination represents a novel means of expressing antigen *in vivo* for the generation of both humoral and cellular immune responses. The present invention uses this technology to elicit protective immunity against autoimmune diseases as exemplified by the experimental autoimmune encephalomyelitis (EAE), a T cell mediated autoimmune disease of the central nervous system that serves as an experimental model for multiple sclerosis.

RT-PCR verified by Southern blotting and sequencing of PCR products of four different C-C chemokines: MIP-1α , MCP-1, MIP-1β and RANTES was performed on brain samples from EAE rats to evaluate mRNA transcription at different stages of disease. Each PCR product was then used as a construct for naked DNA vaccination. The subsequent *in vivo* immune response to MIP-1α or MCP-1 DNA vaccines prevented EAE, even if disease was induced two months after administration of naked DNA vaccines. In contrast, administration of the MIP-1β naked DNA significantly aggravated the disease. Generation of *in vivo* immune response to RANTES naked DNA had no notable effect on EAE. MIP-1α, MCP-1 and MIP-1β mRNA transcription in EAE brains peaked at the onset of disease and declined during its remission, whereas RANTES transcription increased in EAE brains only following recovery.

Immunization of CFA without the encephalitogenic epitope did not elicit the anti C-C chemokine regulatory response in DNA vaccinated rats. Thus, modulation of EAE with C-C chemokine DNA vaccines is dependent targeting chemokines that are highly transcribed at the site of inflammation at the onset of disease.

We further demonstrate herein that EAE rats display a significantly increased TNF-α specific antibody titer as compared to rats immunized in hind foot pads with Complete Freund's Adjuvant (CFA) alone. A positive correlation in time course between the elevated expression TNF-α at the CNS and the production of anti-self antibodies to this proinflammatory cytokine was observed. This natural immunity to TNF-α could not block the development of disease. An administration of TNF-α naked DNA vaccine, even two months before active induction of disease, enhanced the development of *in vivo* immune response to self TNF-α and conferred EAE resistance. Immunization of CFA without the encephalitogenic epitope, even though induced a local inflammatory process, did not elicit the anti TNF-α regulatory response in DNA vaccinated rats. These anti-self antibodies were found capable of inhibiting the development of disease when transferred to other EAE rats. Thus, modulation of EAE with TNF-α vaccines is dependent targeting cytokine that are highly transcribed at the site of inflammation during the course of disease and therefore provides a tool by which the immune system is encouraged to elicit anti-self protective immunity to restrain its own harmful reactivity only when such a response is needed.

According to the present invention there is thus provided a method for treating a mammal for inducing protective immunity against an autoimmune disease, the method comprising the step of administering to the mammal a therapeutic composition including a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic

acid sequence being operatively linked to one or more transcription control sequences.

According to the present invention there is further provided a method for treating a mammal for inducing protective immunity against an autoimmune disease, the method comprising the steps of (a) removing cells of the mammal; (b) transducing the cells in vitro with a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences; and (c) reintroducing the transduced cells to the mammal.

According to still further features in the described preferred embodiments the transduced cells are reintroduced to the mammal parenterally.

According to the present invention there is further provided a pharmaceutical composition comprising (a) a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences; and (b) a pharmaceutically acceptable carrier.

According to further features in preferred embodiments of the invention described below, the pharmaceutically acceptable carrier is selected from the group consisting of an aqueous physiologically balanced solution, an artificial lipid-containing substrate, a natural lipid-containing substrate, an oil, an ester, a glycol, a virus and metal particles.

According to still further features in the described preferred embodiments the composition is useful for treating an autoimmune disease.

According to still further features in the described preferred embodiments the composition is suitable for parenteral administration to a human.

According to still further features in the described preferred embodiments the pharmaceutically acceptable carrier comprises a delivery vehicle that delivers the nucleic acid sequences to the mammal.

According to still further features in the described preferred embodiments

the delivery vehicle is selected from the group consisting of liposomes, micelles, and cells.

According to the present invention there is further provided an antibody raised against a cytokine expressed by cells transduced with a recombinant construct including an isolated nucleic acid sequence encoding the cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences.

According to further features in preferred embodiments of the invention described below, the autoimmune disease is multiple sclerosis.

According to still further features in the described preferred embodiments the cytokine is a chemokine or tumor necrosis factor alpha.

According to still further features in the described preferred embodiments the chemokine is a C-C chemokine.

According to still further features in the described preferred embodiments the C-C chemokine is selected from the group consisting of macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES).

According to still further features in the described preferred embodiments the transcription control sequences are selected from the group consisting of RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

According to still further features in the described preferred embodiments the recombinant construct is an eukaryotic expression vector.

According to still further features in the described preferred embodiments the recombinant construct is selected from the group consisting of pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives.

According to still further features in the described preferred embodiments the mammal is selected from the group consisting of humans, dogs, cats, sheep, cattle, horses and pigs.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel means to combat the incurable and poorly treatable disease -- multiple sclerosis -- devoid of the limitations associated with protective immunity via administered antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIGs. 1a-d show the dynamics of mRNA transcription of various C-C chemokines in inflamed brains. Rats were injected with 10^7 L68-86 cells to develop transferred EAE (Figure 1a). Before adoptive transfer of disease (day 0), and at various time points: before the onset of disease (day 3), at the day of onset (day 5), the peak (day 7), following recovery (day 10), and 10 days after recovery (day 20) mid-brain and brain stem samples from six different rats at each time point were examined. mRNA was isolated from each sample and subjected to RT-PCR analysis using specific oligonucleotide primers constructed to RANTES, MIP-1α , MIP-1β and MCP-1. Each amplification was, calibrated to β-actin and verified by Southern bolting analysis. Figure 1b shows a representative Southern blot analysis of each time point. Rats were immunized with p68-86/CFA and developed active EAE (Figure 1c). Before the induction of disease (day 0), and at various time points: before the onset of disease (day 8), at the peak (day 13) and 5 days after recovery (day 21) mid-brain and brain stem

samples from six different rats at each time point, were obtained and subjected to RT-PCR as described above. Figure 1d shows a representative Southern blot analysis from each time point.

FIGs. 2a-b demonstrate the prevention of EAE using C-C chemokine naked DNA vaccines. Rats were immunized weekly (three repeated immunizations Figure 2a, five repeated immunizations Figure 2b) with cloned PCR products of various C-C chemokines ligated into a pcDNA3 eukaryotic expression vector, or with the pcDNA3 vector alone, or with PBS. Two weeks after the last immunization all rats were immunized with p68-86/CFA to induce active EAE. One month after the last immunization all rats were immunized with p68-86/CFA to induce active EAE and monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, front and hind limb paralysis. Results are shown as mean clinical score of six rats in each group ± SE.

FIGs. 3a-g show that MIP1-α and MCP-1 naked DNA vaccines decreases CNS mononuclear cell infiltration. When active EAE attained its maximal clinical severity (day 12, second experiment, Figure 2b), samples from the lower thoracic and lumbar regions of the spinal cord were histologically evaluated. Histological scores were determined using an 0 to 3 scale as described in the methods. The mean clinical score ± SE were calculated from 6 sections per spinal cord of 2 representative rats from each group (see Table 2 below for more details).

FIGs. 4a-d show synchronic protective immunity to EAE following C-C chemokine naked DNA vaccination. Twelve days after active induction of disease (with p68-86/CFA) sera of rats from the second experiment (Figure 2b), as well as sera from rats that received the same subsequent set of naked DNA vaccinations, but were finally challenged with the emulsion of PBS and CFA without p68-86, or from rats that received the same subsequent set of naked DNA vaccinations but were

never challenged with p68-86/CA or CFA were tested for antibody titer against each of the four C-C chemokines: MCP-1 (Figure 4a), MIP-1α (Figure 4b), MIP-1β (Figure 4c) and RANTES (Figure 4d). The assay conditions and data calculation of each test was done according to (14). Results are shown as mean $\log_2$ of four different samples ± SE.

FIGs. 5a-d show the kinetics of antibody production in sera of EAE rats following C-C chemokine naked DNA vaccination. Rats were immunized weekly (three repeated immunizations) with the cloned PCR products of various C-C chemokines ligated into a pcDNA3 eukaryotic expression vector as described under Figure 2a. Two weeks after the last immunization all rats were immunized with p68-86/CFA to induce active EAE. At different time points (0, 3, 5, 7, 10, 12, 21, 30 and 40 days after EAE induction) generation of anti-self antibody titer was determined as described under Figures 4a-d. Results are shown as mean $\log_2$ of four different samples ± SE.

FIGs. 6a-d show vaccination with MCP-1 DNA elicited a significant cross-reactive immune response to MIP-1α. Twelve days after active induction of disease, sera of rats from the second experiment that were immunized with various C-C chemokine DNA vaccines and then challenged with p68-86/CFA (Figures 2b and 4a-d) were tested for the development of a cross reactive antibody titer between each of the four C-C chemokines: MIP-1α (Figure 6a) MCP-1 (Figure 6b), MIP-1β (Figure 6c), and RANTES (Figure 6d). Results are shown as mean $\log_2$ of four different samples ± SE.

FIG. 7 show anti chemokine antibodies produced by DNA vaccination provide subsequent protection from severe EAE. Six groups of six rats were immunized with p68-86/CFA to develop active EAE. Four days before the onset of disease, rats were daily challenged (intravenously, days 6-13) with 100 μg of each of neutralizing antibodies (IgG fraction, protein G purification) purified from sera of rats that were previously

vaccinated with various naked DNA vaccines, and were then subjected to active induction of EAE as described under Figure 2a. Purified IgG fraction from rats that were, or were not, vaccinated with pcDNA3 and then subjected to active induction of disease, as well as sera from control rats that were, or were not, subjected to active induction of EAE were all used as controls. EAE was monitored daily by an observer blind to the treatment protocol. Results are shown as mean clinical score of six rats in each group ± SE.

FIGs. 8a-c show the dynamics of mRNA transcription of TNF-α in the inflamed brain of transferred EAE rats. Rats were injected with 10^7 L68-86 cells to develop transferred EAE (Figure 8a). Before adoptive transfer of disease (day 0), and at various time points: before the onset of disease (day 3), at the day of onset (day 5), the peak (day 7), following recovery (day 10), and 10 days after recovery (day 20), mid-brain and brain stem samples from six different rats at each time point were examined. mRNA was isolated from each sample and subjected to RT-PCR analysis using specific oligonucleotide primers constructed to TNF-α (Figure 8b). Each amplification was, calibrated to β-actin (Figure 8c) and verified by Southern bolt analysis (Figures 8b-c).

FIGs. 8d-f show the dynamics of mRNA transcription of TNF-α in the inflamed brain of active EAE rats. Rats were immunized with p68-86/CFA and developed active EAE (Figure 8d). Before the induction of disease (day 0), and at various time points: before the onset of disease (day 8), at the peak (day 13) and 5 days after recovery (day 21) mid-brain and brain stem samples from six different rats at each time point, were obtained and subjected to RT-PCR as described with respect to Figures 8b-c. The results are shown in Figure 8e-f, respectively.

FIG. 9 shows that naked DNA encoding TNF-α augments transcriptionally regulated protective immunity. Twelve days after active induction of disease (with p68-86/CFA) sera of rats from experiment

described in legend to Figure 10, as well as sera from rats that received the same subsequent set of naked DNA vaccinations, but were finally challenged with the emulsion of PBS and CFA without p68-86, or from rats that received the same subsequent set of naked DNA vaccinations but were never challenged with p68-86/CA or CFA, were tested for antibody titer against TNF-α The assay conditions and data calculation of each test was done according to (14). Results are shown as mean log2 of four different samples ± SE. Ab - antibody.

FIG. 10 shows prevention against EAE using TNF-α naked DNA vaccine. Rats were immunized weekly with the cloned PCR products of TNF-α ligated into a pcDNA3 eukaryotic expression vector, or with the pcDNA3 vector alone, or with PBS. Two months after the last immunization all rats were immunized with p68-86/CFA to induce active EAE and were monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, front and hind limb paralysis. Results are shown as mean clinical score of six rats in each group ± SE.

FIG. 11 shows the kinetics of antibody production in sera of EAE rats following TNF-α naked DNA vaccination. Rats were immunized weekly (three repeated immunizations) with the cloned PCR products of TNF-α ligated into a pcDNA3 eukaryotic expression vector. Two months after the last immunization all rats were immunized with either p68-86/CFA to induce active EAE, or with CFA alone. At different time points generation of anti-self antibody titer was determined as described under Figure 9. Results are shown as mean $\log_2$ of four different samples ± SE.

FIG. 12 shows that TNF-α specific antibodies produced by DNA vaccination provide subsequent protection from severe EAE. Six groups of six rats each were immunized with p68-86/CFA to develop active EAE. Four days before the onset of disease, rats were daily challenged (intravenously, days 6-13) with 100 μg of each of TNF-α specific

neutralizing antibodies (IgG fraction, protein G purification) purified from sera of rats that were previously vaccinated with various naked DNA vaccines, and were then subjected to active induction of EAE as described under Figure 10. Purified IgG fraction from rats that were, or were not vaccinated with pcDNA3 and then subjected to active induction of disease, as well as sera from control rats that were, or were not subjected to active induction of EAE were all used as controls. EAE was monitored daily by an observer blind to the treatment protocol. Results are shown as mean clinical score of six rats in each group ± SE.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of DNA vaccines and the use of same to induce protective immunity against autoimmune diseases in mammals. Specifically, the present invention can be used to induce protective immunity against multiple sclerosis by vaccinating with DNA encoding, cytokines, C-C chemokines and tumor necrosis factor alpha in particular.

The principles and operation of the vaccines according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

An ideal way of treating a disease caused by a malfunction of the immune system in distinguishing self from foreign, would be by encouraging this system to elicit self protective immunity and thus restrain its own harmful reactivity to times when such a response is needed. This

task has been achieved in the current study using the novel technology of DNA vaccination.

We have previously used RT-PCR verified by Southern blotting analysis to follow the trafficking of T cells to the site of inflammation during the course of transferred EAE and distinguished between selective and non-selective stages in leukocyte homing to the CNS (3). Based on these data we have described the development of EAE as a sequential event in which a primary influx (days 0-2) activates the blood brain barrier to allow accumulation of a secondary influx of endogenous leukocytes and the initiation of the disease (days 5-9) (3). Using the same experimental system and the same strategy we now show, in one aspect, a positive correlation in time course between the accumulation of the secondary influx at the site of inflammation (3) and an elevated expression of MIP-1α ,MCP-1 and MIP-1β at the site of inflammation. Each of the above C-C chemokines is well known for its competence to attract monocytes and T cells to a site of inflammation and for its ability to elicit the expression of various adhesion molecules that mediate the trafficking of these cells (3). Thus, the positive correlation in time course between chemokine expression and cell accumulation at the target organ may be explicated by the putative biological functions of these chemokines. Unexpectedly, RANTES transcription augmented in EAE brains only after recovery. While similar results were previously obtained in a murine model of the disease (40), the biological implications of this observation are not fully understood.

As detailed in the Examples section hereinunder, MIP-1α or MCP-1 DNA vaccines prevented EAE. MIP-1β naked DNA significantly aggravated the disease, whereas the generation of *in vivo* immune response to RANTES naked DNA had no notable effect on EAE manifestation. Thus, intervention in EAE development by C-C chemokine DNA vaccines was effective only for those chemokines which were highly transcribed during the development of the inflammation. This emphasizes the pivotal

role of these chemokines in the pathogenesis of EAE. It is possible that RANTES plays a role in the establishment and maintenance of the resistant state following recovery.

DNA vaccines represent a novel means of expressing antigens *in vivo* for the generation of both humoral and cellular immune responses (10, 14, 41-43) This technology has proven successful in obtaining immunity not only to foreign antigens and tumors, but also to self antigens, such as a T cell receptor V genes (17) or autologous cytokines (42). C-C chemokines were selected as candidates for DNA vaccination mostly because of their well established role in cell migration to a target organ (22, 23, 44-49). Since DNA vaccination elicits both cellular and humoral responses against products of a given construct (10, 14, 41-43), it is difficult to know which of these responses contributed more to the development of EAE resistance in MCP-1 and MIP-1α DNA vaccinated rats. It has, however, been shown that rabbit anti-MIP-1α antibodies were capable of blocking EAE in a murine model (33), and an antagonist of MCP-1 markedly inhibited arthritis in the MRL-lpr mouse (34). Under our experimental conditions, vaccination with MCP-1 DNA elicited a significant cross-reactive immune response to MIP-1α. Our data clearly show that anti-chemokine antibodies produced by naked DNA vaccination are neutralizing antibodies and can provide subsequent protection from severe EAE. Thus, it conceivable that these antibodies contributes to disease inhibition by in MIP-1α and MCP-1 naked DNA vaccinated rats.

As already mentioned in the Background section hereinabove, a major disadvantage in treating chronic diseases with xenogenic neutralizing antibodies lies in their immunogenicity.

The therapeutic strategy suggested herein, is of advantage over the above methods since it resulted in the generation of immunity to autologous antigens. In addition, the data presented herein reveals an unexpected, yet extremely important, advantage in applying C-C chemokine DNA

vaccination. It appears that the immune response to each of the given DNA constructs elicited only during the course of disease and only at the time when the transcription of the related chemokine profoundly elicited at the site of inflammation EAE induction.

Finally, a recent study shows a coordinated chemokine up-regulation in brain and spinal cord during clinical relapse in mice with relapsing EAE (52). This emphasizes the importance of treating a disease caused by a malfunction of the immune system in distinguishing self from foreign, such as multiple sclerosis, by encouraging this system to elicit anti-self protective immunity and thus restrain its own harmful only when such a response is needed.

In the process of negative selection in the thymus many, but not all, self reactive T cells are eliminated. Autoreactive T cells that escape thymic selection can be identified in both healthy individuals and those suffering form self destructive autoimmune diseases (72). In healthy individuals self tolerance is maintained in part through mechanisms acting outside the thymus that keep these autoreactive lymphocytes under control. Anti inflammatory cytokines such as TGF-β, IL-10, IL-4 and IL-13 produced by antigen specific regulatory T cells and macrophages are involved in restraining the activity of autoreactive T cells, and for keeping the tolerant state under control (73-82).

In another aspect, the present invention demonstrates, for the first time, the appearance of 'natural' anti self antibodies to a key proinflammatory cytokine, TNF-α, during the development of a T cell mediated autoimmune disease of the central nervous system. These antibodies were developed in rats immunized with p68-86/CFA and not with the CFA alone even though both groups exhibited an extensive local inflammatory process at the site of CFA immunization. Thus, only the transcription of the inflammatory cytokine TNF-α at an privileged

autoimmune site (CNS) enabled the triggering of an anti-self response against this pro-inflammatory cytokine.

The biological significance of these results is apparent. An ideal immune system would be evolutionary selected to centralize its destructive competence against invading microbes rather than the self tissues its was designed to protect (83-85). The underlying mechanism by which the immune system distinguishes a gene products transcribed at a privileged autoimmune site from the same gene product transcribed at a local site of inflammation is, however elusive. A partial explanation has been previously suggested by C. C. Goodnow and his colleagues who demonstrated that peripheral clonal exclusion of self reactive B cells occurs at germinal centers of lymph nodes that drain tissues lacking immune prevalence, where competition for follicular niches do not exclude self reactive cells from the recalculating B cell repertoire (86). The 'natural' anti self production to TNF-α in EAE susceptible rats was, however, not sufficient to prevent the development of an autoimmune condition (6/6 sick rats).

An ideal way of treating a disease caused by a malfunction of the immune system in distinguishing self from foreign would be by encouraging this system to elicit self protective immunity and thus restrain its own harmful reactivity only when such a response is needed. This task has been achieved according to the present invention using the novel technology of naked DNA vaccination. DNA vaccines represent a novel means of expressing antigens *in vivo* for the generation of both humoral and cellular immune responses (10, 14, 41-43). This technology has proven successful in obtaining immunity not only to foreign antigens and tumors, but also to self antigens, such as a T cell receptor V genes (17) or autologous cytokines (42). Since DNA vaccination elicits both cellular and humoral responses against products of a given construct (10, 14, 41-43), it is difficult to know which of these responses contributed more to the

development of EAE resistance in TNF-α DNA vaccinated rats. The data showing that TNF-α specific self antibodies produced by naked DNA vaccination can provide subsequent protection from severe EAE votes for there pivotal role in the prevention of EAE. The mechanism by which TNF-α specific naked DNA vaccines augment production of anti-self neutralizing antibodies is not fully addressed yet. The possibility that naked DNA vaccination elicits the activation of self reactive T cells that help production of autoreactive antibodies to TNF-α when this cytokine is profoundly transcribed at an autoimmune privileged area is not excluded.

As already mentioned, from a clinical perspective, however, a major disadvantage in treating chronic diseases with xenogenic neutralizing antibodies lies in their immunogenicity. This has motivated investigators to develop chimeric humanized antibodies (reviewed in (50)), and monoclonal antibodies engineered with human Ig heavy and light chain yeast artificial chromosome (YAC) (51). However, following repeated immunization, these engineered antibodies do trigger an apparently allotypic response. The therapeutic strategy suggested by the present invention, is of advantage over the above methods since it resulted in the generation of immunity to autologous antigen only during the course of disease at the time when the transcription of the proinflammatory cytokine profoundly elicited at the site of inflammation.

Thus, in accordance with one aspect of the present invention, there is provided a method for treating a mammal for inducing protective immunity against an autoimmune disease. According to the method, a mammal is administered with a therapeutic composition including a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences.

As used herein in the specification and in the claims section below, the phrase "inducing protective immunity" refers to eliciting neutralizing antibodies via DNA vaccination.

As used herein in the specification and in the claims section below, the phrase "autoimmune disease" refers to a disease resulting from a disordered immune reaction in which antibodies are produced that damage components of one's own body.

As used herein in the specification and in the claims section below, the terms "cytokine" and "chemokine" also refer to therapeutically effective potions of cytokines and chemokines, i.e., portions that are effective in eliciting the described protective immunity.

Thus, in accordance with another aspect of the present invention, there is provided a method for treating a mammal for inducing protective immunity against an autoimmune disease. The method according to this aspect of the invention is effected by executing the following method steps, in which, in a first step, cells are removed of the mammal, in a second step, the cells are transduced in vitro with a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences, whereas in a third step, the transduced cells are reintroduced to the mammal.

As used herein in the specification and in the claims section below, the term "transduced" or "transducing" refers to the result of a process of inserting nucleic acids into cells. The insertion may, for example, be effected by transformation, viral infection, injection, transfection, gene bombardment, electroporation or any other means effective in introducing nucleic acids into cells. Following transduction the nucleic acid is either integrated in all or part, to the cell's genome (DNA), or remains external to the cell's genome, thereby providing stably transduced or transiently transduced cells.

The cells according to this method may be of any kind. Especially suitable cells are those readily removable, tranduceable, and reintroduceable cells, such as, but not limited to, cells of the various blood lineage, derived either from whole blood or bone marrow, fibroblast cells, etc. The transduced cells are preferably reintroduced to the mammal parenterally.

According to yet another aspect of the present invention there is provided a pharmaceutical composition suitable for effecting the above methods of the present invention. The composition includes a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences, and a pharmaceutically acceptable carrier.

The pharmaceutically acceptable carrier may be of any acceptable form. Examples include, but are not limited to, aqueous physiologically balanced solutions, artificial lipid-containing substrates, natural lipid-containing substrates, oils, esters, glycol, viruses and metal particles.

The composition is preferably made suitable for parenteral administration to a human. It is therefore preferably sterile (except for infective particles, if deliberately present therein) and may additionally include adjuvant allowed for use in human beings, such as Bacillus Calmette Guerein (BCG) including adjuvant.

According to one embodiment of the present invention, the pharmaceutically acceptable carrier includes a delivery vehicle that delivers the nucleic acid sequences to the mammal. Suitable delivery vehicles include, but are not limited to, liposomes, micelles, and cells.

The construction, operation and use of the above pharmaceutically acceptable carriers for DNA vaccination and the above delivery vehicles are described in detail in U.S. Pat. No. 5,705,151 to Dow *et al.*, entitled "gene therapy for T cell regulation", which is directed at anti-cancer treatment, and is hereby incorporated by reference as if fully set forth herein.

Thus, for therapeutic or prophylactic treatment, the composition according to the present invention may include thickeners, carriers, buffers, diluents, surface active agents, preservatives, and the like, all as well known in the art. Pharmaceutical compositions may also include one or more active ingredients, such as ,but not limited to, anti-inflammatory agents, anti-microbial agents, anesthetics and the like.

The pharmaceutical composition may be administered in either one or more of ways. Administration may be effected topically (including ophtalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip or intraperitoneal, subcutaneous, or intramuscular injection.

Formulations for topical administration may include but are not limited to lotions, ointments, gels, creams, suppositories, drops, liquids, sprays and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Formulations for parenteral administration may include but are not limited to sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Dosing is dependent on responsiveness, but will normally be one or more doses per week or month, with course of treatment lasting from several weeks to several months. Persons ordinarily skilled in the art can easily determine optimum dosages, dosing methodologies and repetition rates.

According to yet another aspect of the present invention there is provided an antibody raised against a cytokine expressed by cells transduced with a recombinant construct including an isolated nucleic acid sequence encoding the cytokine, the nucleic acid sequence being operatively linked to one or more transcription control sequences.

As used herein in the specification and in the claims section below, the term "antibody" refers to any monoclonal or polyclonal

immunoglobulin, or a fragment of an immunoglobulin such as sFv (single chain antigen binding protein), Fab1 or Fab2. The immunoglobulin could also be a "humanized" antibody, in which murine variable regions are fused to human constant regions, or in which murine complementarity-determining regions are grafted onto a human antibody structure (Wilder, R.B. et al., J. Clin. Oncol., 14:1383-1400, 1996). The terms "sFv" and "single chain antigen binding protein" refer to a type of a fragment of an immunoglobulin, an example of which is sFv CC49 (Larson, S.M. *et al.*, Cancer, 80:2458-68, 1997).

As further exemplified in the Examples section hereinunder, the methods, compositions and antibodies according to the present invention are useful at inducing protective immunity against autoimmune diseases, multiple sclerosis, in particular.

According to a preferred embodiment of the present invention the nucleic acid sequence selected encodes a chemokine, in particular a C-C chemokine, most particularly inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and/or regulation on activation normal T expressed and secreted (RANTES). Any combination of sequences encoding cytokines may be simultaneously employed according to the present invention on different or single constructs. According to another embodiment the nucleic acid sequence selected encodes tumor necrosis factor alpha.

The transcription control sequences may be of any suitable type compatible with eukaryotic gene expression. Strong and effective control sequences are preferably of choice. These sequences can be from a mammalian or viral source. Examples include, but are not limited to, RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences, all of which are potent and effective control sequences, capable of efficiently directing gene expression.

According to a preferred embodiment of the present invention the recombinant construct is an eukaryotic expression vector, such as, but not limited to, pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, which are available from Invitrogen, pCI which is available from Promega, pBK-RSV and pBK-CMV which are available from Stratagene, pTRES which is available from Clontech, and their derivatives.

The present invention is suitable for prevention autoimmune diseases in any mammal. Examples include, but are not limited to, humans, dogs, cats, sheep, cattle, horses and pigs.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Materials and Experimental Methods

Rats: Female Lewis rats, approximately six weeks old were purchased from Harlan (Israel) and maintained under SPF conditions in an animal facility.

Peptide antigens: Myelin basic protein (MBP) p68-86, YGSLPQKSQRSQDENPV (SEQ ID NO:1), was synthesized on a MilliGen 9050 peptide synthesizer by standard 9-fluorenylmethoxycarbonyl chemistry. Peptides were purified by high performance liquid chromatography. Structure was confirmed by amino acid analysis and mass spectroscopy. Only peptides that were greater than 95 % pure were further used.

Immunizations and active disease induction: Rats were immunized subcutaneously in the hind foot pads with 0.1 ml of MBP epitope 68-86 (p68-86) dissolved in phosphate buffer saline (PBS, 1.5 mg/ml) and emulsified with an equal volume of CFA (incomplete Freund's adjuvant supplemented with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra

in oil (Difco laboratories, Inc., Detroit, MI). Rats were then monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, front and hind limb paralysis.

T-cell lines: Nine days after induction of active EAE, draining lymph node cells (DLNC) were cultured ($12x10^6$/ml) for three days in stimulation medium that includes Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with β-mercaptoethanol ($5x10^{-5}$ M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 μg/ml) streptomycin (100 μg/ml), 1 % syngeneic serum and 20-30 μg/ml of the immunizing epitope, washed and resuspended in resting medium which was identical to the stimulation medium without syngeneic serum and with the addition of 10 % fetal calf serum (Gibco BRL) and 12.5 % supernatant of Con A stimulated splenocytes as a source of T cell growth factors. Con A supernatant was prepared as described elsewhere (36). After five-seven days in resting medium the cells ($5x10^5$/ml) were activated for three days in the presence of irradiated (2500R) syngeneic thymocytes ($12x10^6$/ml) and 10-20 μg/ml of p68-86. The activated T cells were then either used for induction of transferred EAE or resuspended in resting medium for additional growing cycles.

Induction of transferred EAE: Transferred EAE was induced by immunizing Lewis rats (intraperitoneally) with 10^7 in vitro activated (day 3) L68-86 cells.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis: RT-PCR analysis, verified by Southern blotting, was utilized on brain samples according to a protocol described elsewhere with some modifications (3). Rats were euthanized by CO_2 narcosis. Brain samples containing mainly the midbrain and brain stem were obtained after perfusion of the rat with 160-180 ml of ice-cold phosphate buffer saline (PBS) injected into the left ventricle following an incision in the right

atrium. Each sample was homogenized. Total RNA was extracted using the Tri-Zol procedure (Gibco BRL) according to the manufacturer's protocol. mRNA was then isolated using a mRNA isolation kit (# 1741985 Boheringer Mannheim, Germany), and reverse transcribed into first strand cDNA exactly as is describe in detail elsewhere (3). First strand cDNA was then subjected to 35 cycles of PCR amplification using specific oligonucleotide primers which were designed based on the published sequence of each cytokine (NCBI accession numbers: Rat MIP-1α U06435, Rat MIP-1β U06434, Rat RANTES U06436 and Rat MCP-1 M57441, Rat TNF-α L00981, which sequences are incorporated by reference as if fully set forth herein) as follows:

MIP-1α sense: 5'-ATGAAGGTCTCCACCACTGCCCTTGC-3' (SEQ ID NO:2); MIP-1α antisense: 5'-TCAGGCATTCAGTTCCAGCTCAGTG-3' (SEQ ID NO:3); MIP-1β sense: 5'-ATGAAGCTCTGCGTGTCTGCCTTC-3' (SEQ NO:4); MIP-1β antisense: 5'-TCAGTTCAACTCCAAGTCATTCAC-3' (SEQ ID NO:5); RANTES sense: 5'-ATGAAGATCTCTGCAGCTGCATCC-3' (SEQ ID NO:6); RANTES antisense: 5'-CTAGCTCATCTCCAAATAGTTG-3' (SEQ ID NO:7); MCP-1 sense: 5'-ATGCAGGTCTCTGTCACGCTTCTGGGC-3' (SEQ ID NO:8); MCP-1 antisense: 5'-CTAGTTCTCTGTCATACTGGTCAC-3' (SEQ ID NO:9); TNF-α sense: 5'-ATGAGCACAGAAAGCATGAT-3' (SEQ ID NO:10); and TNF-α antisense: 5'-TCACAGAGCAATGACTCCAAA -3' (SEQ ID NO:11).

All RNA samples were calibrated to Rat β-actin: β-actin sense 5'-CATCGTGGGCCGCTCTAGGCA-3' (SEQ ID NO:11); and β-actin antisense: 5'-CCGGCCAGCCAAGTCCAGACG-3' (SEQ ID NO:12).

The cycle profile was: denaturation at 95 °C for 60 sec, annealing at 55 °C for 60 sec, and elongation at 72 °C for 60 sec.

Amplified products were subjected to agarose gel electrophoresis, transferred to a nylon membranes (MagnaGraph nylon transfer membrane, msi, Westborough, MA), fixed with ultraviolet light (120 mjoules) and hybridized with 10^6 cpm/ml of $\alpha^{32}P$ labeled DNA fragments encoding the full length PCR product of each cytokine and of β-actin (random priming: Amersham, Arlington Heights, IL). PCR products were used as probes only after each PCR product was cloned and its sequence was verified as further described hereinunder.

Cloning and sequencing of PCR products: Each of the amplified PCR products described above was cloned into a pUC57/T vector (T-cloning Kit #K1212, MBI Fermentas, Lithuania) and transformed to *E. coli* according to the manufacturer's protocol. Each clone was then sequenced (Sequenase version 2, USB, Cleveland, Ohio) according to the manufacturer's protocol. PCR products were selected to be used as constructs for naked DNA vaccination only after cloning and sequence verification.

DNA vaccination: DNA vaccination was performed according to Waisman *at al.* with some modifications (17). Sequenced PCR products of rat MIP-1α, MCP-1, MIP-1β, RANTES and TNF-α were transferred into a pcDNA3 vector (Invitrogen, San Diego, CA). Large scale preparation of plasmid DNA was conducted using Mega prep (Qiagen Inc., Chatsworth, CA). Cardiotoxin (Sigma, St. Louis, MO) was injected into the tibialis anterior muscle of 6-8 weeks old female Lewis rats (10 μM per leg). One week following injection rats were injected with 100 μg DNA in PBS. Four-five days after the first immunization one rat from each group was sacrificed and transcription of the relevant chemokine was verified using RT-PCR on tibialis anterior muscle samples. Thereafter, naked DNA vaccines were given 3-5 times with intervals of 6-7 days between each injection.

Production and purification of recombinant proteins: PCR products were recloned into a PQE expression vector (PQE-30, PQE-31 or PQE-32 according the correct open reading frame) and were expressed in *E. Coli* (Qaigen, Chatsworth, CA) and then purified by a NI-NTA-supper flow affinity purification of 6xHis proteins (Qaigen, Chatsworth, CA). Each recombinant protein sequence has then been verified (N-terminus) by the AB1494 Sequencer (Applied Biosystem Division, Perkin Elmer).

Purification of antibodies: Antibodies from rat sera were purified using a High-Trap Protein G column (Pharmacia, Piscataway, NJ) according the manufacturer's protocol. Then antibody titer to various chemokines was determined by an ELISA assay as described bellow.

In vitro chemotaxis assay: In vitro chemotaxis assay was conducted as previously described (37) with minor modifications according to (38). Peritoneal macrophages were isolated as previously described (38) and suspended in DMEM enriched with 1 % BSA. Cell migration was evaluated in standard Boyden chambers (Neuroprobe, Cabin John; MD). Macrophages ($1.2x10^6$ cells) were added to the upper well. Chemotactic factors: fMLP (Sigma, 10^{-7} M), rat recombinant MIP-1α (Chemicon International, Temecula, CA, 200 ng/ml) or rat recombinant MCP-1 (Chemicon International, Temecula, CA, 100 ng/ml) were added to the lower wells, with, or without pre-incubation with the required antibodies (10 μg/well) at 37 °C for 30 minutes. Migration was allowed to proceed for 90 minutes at 37 °C. The celloulose nitrate filters (5 μm pore size) were then fixed and stained as described (37). Five x 400 fields were selected randomly on each filter and the migrating cells were counted.

Evaluation of anti-chemokine antibody titer in sera of DNA vaccinated rats: A direct ELISA assay was utilized to determine the anti-C-C chemokine antibody titer in DNA vaccinated rats. Each recombinant chemokine which was produced, as well as commercial recombinant rat RANTES, rat MIP-1α, rat MCP-1, human MIP-1β (Chemicon International,

Temecula, CA) and rat TNF-α (Genzyme, Cambridge, MA) were coated onto 96 well ELISA plates (Nunc, Denmark), at concentrations of 50 ng/well. Rat anti-sera, in serial dilutions from 2^8 to 2^{30} were added to ELISA plates coated with each recombinant chemokine. Goat anti-rat IgG alkaline phosphatase conjugated antibodies (Sigma) were used as a labeled antibody. p-Nitrophenyl Phosphate (p-NPP, Sigma) was used as a soluble alkaline phosphatase substrate. The assay conditions and data calculation of each test were done according to (14). Results are shown as $\log_2$ antibody titer ± SE. Commercial monoclonal antibodies to each cytokine (Chemicon International) were used as a positive control for detected sera in each experiment.

Histopathology: Histological examinations of hematoxylin and eosin-stained sections of formalin-fixed, paraffin-embedded sections of brain and the lower thoracic and lumbar regions of the spinal cord were performed. Each section was evaluated without knowledge of the treatment status of the animal. The following scale was used: 0, no mononuclear cell infiltration; 1, 1 to 5 perivascular lesions per section with minimal parenchymal infiltration; 2, 5 to 10 perivascular lesions per section with parenchymal infiltration; and 3, >10 perivascular lesions per section with extensive parenchymal infiltration. The mean histological score ± SE was calculated for each treatment group. Representative photomicrograph are shown in Figure 3a-g.

Antigen-specific T cell proliferation assays. Lewis rats were immunized with MBP p68-86/CFA as described above. Nine to ten days later spleen cells were suspended in stimulation medium and cultured in U-shape 96-well microculture plates ($2x10^5$ cells/well) for 72 hours, at 37 °C in humidified air containing 7.5 % CO_2. Each well was pulsed with 2 μCi of [^{3}H]-Thymidine (specific activity 10 Ci/mmol) for the final six hours. The cultures were then harvested on fiberglass filters and the proliferative

response expressed as CPM±SD or as stimulation index (SI) (mean CPM of test cultures divided by mean CPM of control cultures).

Cytokine determination. Spleen cells from EAE donors were stimulated *in vitro* (10^7 cells/ml) in 24 well plates (Nunc) with 100 μM p68-86. After 72 hours of stimulation, supernatants were assayed by semi-ELISA kits, that include antibody pairs and recombinant rat cytokines, as follows: IFN-γ, rabbit anti-rat IFN-γ polyclonal antibody (CY-048, Innogenetics, Belgium) as a capture antibody, biotinylated mouse anti-rat monoclonal antibody (CY-106 clone BD-1, Innogenetics) as a detection antibody, and Alkaline phosphatase-Streptavidin (cat No. 43-4322, Zymed, SF, CA) with rat recombinant IFN-γ as a standard (Cat. No. 3281SA, Gibco BRL); TNF-α, commercial semi-ELISA kit for the detection of rat TNF-α, (Cat. No. 80-3807-00, Genzyme, Cambridge, MA); IL-4, mouse anti-rat IL-4 monoclonal antibody (24050D OX-81, PharMingen, San Diego, CA) as a capture antibody, and rabbit anti-rat IL-4 biotin-conjugated polyclonal antibody (2411-2D, PharMingen) as second antibody. Recombinant rat IL-4 purchased from R&D (504-RL) was used as a standard.

Statistical analysis. Significance of differences was examined using Student's t-test (Figures 2, 4, 5, 9, 10, 11 and 12). A value of $p<0.05$ was considered significant. One way multiple range ANOVA test with significance level of $p<0.05$ was performed for multiple compression of antibody titer to various chemokines in naked DNA vaccinated rats (Figures 4a-d, and 6a-d). Mann-Whitney sum of ranks test was used to evaluate significance of differences in mean of maximal clinical score (Figure 10). Value of $P<0.05$ was considered significant.

Experimental Results

Dynamics of transcription of various C-C chemokine mRNAs in the inflamed brain: Rats injected with L68-86 developed transferred EAE that persisted for 5-6 days (Figure 1a). Before adoptive transfer of disease (day 0), and at various time points: before the onset of disease (day 3), at

the day of onset (day 5), the peak (day 7), following recovery (day 10), and 10 days after recovery (day 20) midbrain-brain stem samples were obtained from six different rats at each time point. From each sample, mRNA was isolated and subjected to RT-PCR analysis using specific oligonucleotide primers which were constructed for each chemokine (SEQ ID NOs:2-9). Each amplification was calibrated to β-actin and verified by Southern blotting analysis. This enabled semi-quantitative analysis of the dynamics of mRNA transcription of each of the above C-C chemokines at the site of inflammation. Figure 1b shows representative results from each time point of the experiment. An increased transcription of MIP-1α ,MCP-1 and MIP-1β mRNA in EAE brains was observed at the onset of disease (day 5). The augmented transcription of MIP-1α and MCP-1 regressed to background within two days even though disease continued to progress to its maximal clinical score on day 7 (Figures 1a-b). The increased transcription of MIP-1β, however, declined to its background in correlation with recovery (Figures 1a-b). Unexpectedly, RANTES transcription increased in EAE brains only after recovery. The biological significance of this observation remains to be elucidated. Rats with developing active disease manifested similar mRNA transcription characteristics as those with developing transferred disease. That is, an elevated expression of MCP-1, MIP-1α and MIP-1β at the onset of disease which declines during recovery, and an augmented transcription of RANTES following recovery (Figures 1c-d).

Prevention of EAE using C-C chemokine naked DNA vaccines: Cloned PCR products of each C-C chemokine, obtained as described above, were ligated into a pcDNA3 eukaryotic expression vector and used as constructs for naked DNA vaccination (Figures 2a-b). In a first experiment (Figure 2a) rats were subjected to three weakly injections of each construct. Control rats were either injected with the pcDNA3 vector alone, or with PBS. Two weeks after the last immunization all rats were immunized with

p68-86/CFA to induce active EAE. All control (PBS immunized) and pcDNA3 vaccinated rats developed active disease that persisted for 5-6 days (Figure 2a, 6/6 in each group with a maximum clinical score 2.33±0.1 in control and 2±0.26 in pcDNA3 immunized rats). In contrast, rats injected with either MIP-1α or MCP-1 DNA naked DNA vaccines were resistant to EAE (incidence of 0/6 for MIP-1α and 1/6 for MCP-1 vaccinated rats with a maximum clinical score of 0 and 0.33±0.34, respectively, $p<0.001$ for each treatment compared with either control or pcDNA3 treatments). Thus, the subsequent *in vivo* immune response to MIP-1α or MCP-1 DNA vaccines prevented EAE. In contrast, administration of the MIP-1β naked DNA significantly aggravated active EAE (Figure 2a, 6/6 in each group maximum clinical score 3.2±0.17 compared with 2.33±0.1 in control and 2±0.26 in pcDNA3 immunized rats, $p<0.033$ and 0.028 respectively).

MIP-1α, MCP-1 and MIP-1β mRNA transcription in EAE brains peaked at the onset of disease and declined during its remission, whereas RANTES transcription increased in EAE brains only following recovery (Figures 1a-d). Thus, intervention in EAE development by C-C chemokine DNA vaccines is effective provided that the related chemokine is highly transcribed at the site of inflammation at the onset of disease.

In a subsequent, second, experiment each of the above constructs, as well as pcDNA3 alone, were administered five rather than three times (Figure 2b). As with the first experiment, MIP-1α and MCP-1 naked DNA vaccines effectively prevented the development of active EAE (incidence of 1/6 for each treatment with a maximum clinical score of 0.17±0.17, compared with 6/6 in either control and pcDNA treated rats, $p<0.001$ for each comparison), MIP-1β vaccine significantly aggravated the disease (Figure 2b, 6/6 in each group maximum clinical score 3±0 compared with 2±0 in control and 1.33±0.21 in pcDNA3 immunized rats, $p<0.001$ for each comparison) and RANTES naked DNA vaccination did not exhibit any

notable effect on disease manifestation. Five consecutive immunizations of pcDNA3 did, however, notably affect disease severity (maximum score in control rats 2±0 compared with 1.33±0.21 in pcDNA treated rats, $p<0.007$). It is possible that numerous subsequent immunizations of an eukaryotic vector with a viral promoter may affect cytokine production by T cells, as has recently been suggested (39).

When active EAE attained its maximal severity (day 12, Figure 2b) spinal cord samples of representative animals from each group (second experiment) were evaluated histologically (Table 1, Figures 3a-g). While control EAE rats and rats previously immunized with pcDNA3 all displayed perivascular lesions with parenchymal mononuclear cell infiltration (Figures 3b-c, and Table 1, b and c, mean histological score 2.2±0.2 and 1.8±0.2, respectively) rats previously immunized with MIP-1α or MCP-1 naked DNA vaccines were either free of mononuclear cell infiltration, or exhibited minimal parenchymal infiltration (Figures 3d-e, and Table 1, d and e, compared with Figures 3b-c, and Table 1, b and c, mean histological score 0.2±0.2 and 0.4±0.24 compared with 1.8±0.2 and 2.2±0.2, $p<0.001$). In contrast, rats that were immunized with MIP-1β naked DNA vaccines manifested an extensive parenchymal mononuclear cell infiltration (Figure 3f and Table 1, f, mean histological score 3±0). Thus, inhibition or exacerbation of disease by various naked DNA vaccines could each be demonstrated histologically.

Table 1

MIP1-α and MCP-1 naked DNA vaccines decreases CNS mononuclear cell infiltration

	Treatment[1]	EAE	Mean Histological Score[2]
a	-	-	0±0
b	-	+	2.2±0.3
c	pcDNA3 alone	+	1.8±0.2
d	pcDNA3/MCP-1	+	0.2±0.2*
e	pcDNA3/MIP-1α	+	0.4±0.24*
f	pcDNA3/MIP-1β	+	3±0**
g	pcDNA3/RANTES	+	1.8±0.2

[1] Rats were treated as described in legend to Figure 2b.

[2]When active EAE attained its maximal clinical severity (day 12, second experiment, Figure 2b), samples from the lower thoracic and lumbar regions of the spinal cord were histologically evaluated. Histological scores were determined using an 0 to 3 scale as described in the methods. The mean clinical score ± SE were calculated from 6 sections per spinal cord of 2 representative rats from each group. * $p<0.001$ for D and E compared with either B or C; ** $p<0.001$ for f compared with either b or c.

Natural autoimmunity to C-C chemokines in EAE is augmented with naked DNA vaccination: The development of anti-self protective immunity in DNA vaccinated rats was evaluated. When active EAE attained its maximal severity (day 12, Figure 2b), blood samples of all animals that were sacrificed for histological evaluation (second experiment, Table 1, Figures 3a-g) were analyzed for the production of antibodies against gene products of each vaccinated DNA (Figures 4a-d), for the kinetics of antibody production along the course of active disease (Figures

5a-d), and for the possible development of cross-reactive immunity between various chemokines (Figures 6a-d).

Rats, without DNA vaccination with developing EAE display a notable anti-self antibody titer to various C-C chemokines (to MCP-1, Figure 4a, 12.25±0.55 Vs. 7±0.47; to MIP-1α, Figure 4b, 11±0.47 Vs. 8.25±0.55; to MIP-1β Figure 4c 11±0.47 Vs. 7±0.47; to RANTES, Figure 4d 11.25±0.55 Vs. 7±0.47) which is, however, not sufficient to prevent the development of disease. Naked DNA vaccination, on the other hand, significantly augmented this antibody titer (Figures 4a-d, 14.5±0.33, 14.75±0.55, 16.25±0.72 and 17±0.66 in rats immunized with MCP-1, MIP-1α, MIP-1β or RANTES constructs Vs. 9±0.47, 8.5±0.33, 8.75± 0.29 and 7±0.47 in rats immunized with pcDNA alone, $p<0.05$ for each reciprocal comparison, and Vs. 7±0.47, 8.25±0.55, 7±0.47 and 7±0.47 in naive rats, $p<0.05$ for each reciprocal comparison, no significant difference was identified between rats immunized with pcDNA alone and naive controls). Nevertheless, the antibody titer in rats immunized with each C-C chemokine DNA except RANTES markedly increased following induction of active EAE, but not following an immunization with CFA without p68-86 (Figures 4a-d, 21.25±0.98 19.25±0.73, 21.25±0.99 and 20±0.47 in rats immunized with MCP-1, MIP-1α, MIP-1β or RANTES constructs and then with p68-86/CFA Vs. 14.75±0.55, 13.75±0.72, 15.25± 0.55 and 17.5±1.1 in rats immunized with MCP-1, MIP-1α, MIP-1β or RANTES constructs and then with CFA, $p<0.05$, for each reciprocal comparison, except for the last one, $0.05 < p < 0.1$).

Thus, naked DNA vaccines may serve as a powerful technique to generate protective immunity against autologous cytokines and provides a tool by which the immune system is encouraged to elicit anti-self protective immunity to restrain its own harmful reactivity only when such a response is needed.

Sera form each of the above groups, immunized with various DNA vaccines and then with p68-86/CFA, were analyzed for a possible development of cross reactive antibody titer (Figures 6a-d). Sera from MIP-1α, MIP-1β and RANTES DNA vaccinated rats manifested a highly specific titer against homologous antigen ($p<0.05$ for the compression of each titer to any of the other 3 chemokines). MCP-1 vaccinated rats, however, exhibited a significant cross reactive antibody titer against MIP-1α (Figure 6b ; 21.25±0.99 to self, 17±0.66 to MIP-1α and 11±0.47 to either MIP-1β or RANTES; $p<0.005$ for the comparison of anti- MIP-1α to anti-MIP-1β or RANTES, antibody titer, and for the comparison of anti-self to anti- MIP-1α antibody titer). Since both MCP-1 and MIP-1α naked DNA vaccines are protective, it is possible that the protective immunity generated by anti- MCP-1 DNA vaccination may be mediated at least in part by reaction with MIP-1α.

Since DNA vaccination elicits both cellular and humoral responses against products of a given construct it is difficult to know which of these responses contributed more to the development of EAE resistance in MCP-1 and MIP-1α DNA vaccinated rats. To evaluate the possible contribution of anti-self antibodies to the development of EAE resistance twelve days after active induction of EAE, when production of anti-self antibodies in naked DNA vaccinated rats attained at its maximal titer (Figures 5a-d), antibodies were purified (IgG fraction, protein G purification) and evaluated for their competence to inhibit the migration of oil-induced peritoneal macrophages in a Boyden chemotaxis chamber assay, as previously described (37). MCP-1 and MIP-1α specific antibodies produced in MCP-1 naked DNA vaccinated rats significantly blocked MCP-1 and MIP-1α induced chemotaxis (Table 2, 70±7 and 88±12 Vs. 185±15, $p<0.001$ for each comparison), whereas MIP-1α specific antibodies generated in MIP-1α naked DNA vaccinated rats effectively blocked MIP-1α induced

chemotaxis (63±4 Vs. 155±15, $p<0.001$), and to a much lesser extent MCP-1 induced chemotaxis (144±11 Vs. 185±15, $p<0.05$, Table 2).

Thus, MCP-1 and MIP-1α chemokine specific antibodies generated in naked DNA vaccinated rats are neutralizing antibodies.

These antibodies were then evaluated for their competence to provide subsequent protection from severe EAE (Figure 7). Four days before the onset of active EAE, rats were daily challenged (days 6-13) with 100 μg of each of the above neutralizing antibodies, or with antibodies from rats that were vaccinated with pcDNA3 alone. Repeated administration of antibodies from MCP-1 and from MIP-1α DNA vaccinated rats provided substantial protection from disease progression (Mean maximal score of 0.66±0.2 in rats treated with purified antibodies from either MCP-1 or MIP-1α DNA vaccinated donors Vs. 3.16±0.2 and 3±0 in rats treated with purified antibodies from PBS or pcDNA3 treated rats, $p<0.001$ for each compression). In addition, elevated levels of MCP-1 and MIP-1α specific antibodies could be observed in spinal cord fluid (SCF) of EAE rats (day 12 of active EAE) that were previously subjected to MCP-1 or MIP-1α naked DNA vaccines ($\log_2$ antibody titer of 27±3 and 18± 2 to MCP-1 and MIP-1α in SCF of rats administered with MCP-1 naked DNA vaccine, and of 25±3 to MIP-1α in SCF of rats administered with MIP-1α naked DNA vaccine, compared to 12±2 and 10±1 in SCF of rats treated with pcDNA3 or PBS, $p<0.01$ for each comparison. Rats administered with MIP-1α naked DNA vaccine did not generate a significant antibody titer to MCP-1 compared with rats administered with pcDNA3 or PBS). Thus, during the course of EAE, neutralizing antibodies to MCP-1 and MIP-1α are generated in MCP-1 and MIP-1α DNA vaccinated rats and elevated levels of these antibodies can be identified at the site of inflammation in the CNS where they probably block disease progression.

Table 2

Antibodies from MIP-1α and MCP-1 naked DNA vaccinated rats block MIP-1α and MCP-1 induced chemotaxis in vitro

	Purified antibodies (IgG) from:				
	-	Control EAE rats	MIP-1α[b] DNA vaccinated EAE rats	MCP1[b] DNA vaccinate(EAE rats	pcDNA3[b] DNA vaccinated EAE rats
Chemo- attractant			(cells/field±SE)		
Medium	60 ± 6	66 ± 8	62 ± 4	57 ± 5	65 ± 6
fMLP (10-7M)	220 ± 14	213 ± 17	215 ± 17	211 ± 17	211 ± 19
MIP-1α (200 ng/ml	155 ± 15	143 ± 10	63 ± 4*	88 ± 12*	144 ± 11
MCP-1 (100 ng/ml)	185 ± 15	179 ± 12	144 ± 11**	70 ± 7*	173 ± 10

Twelve days after active induction of EAE, when production of anti-self antibodies in naked DNA vaccinated rats attained at its maximal titer (Figures 5a-d), antibodies were purified (IgG fraction, protein G purification) and evaluated for their competence to inhibit the migration of oil-induced peritoneal macrophages in a Boyden chemotaxis chamber assay. fMLP (Sigma) was used as a positive control for Chemoattraction. Result are shown as Mean of triplicates ±SE. b Donor rats were treated as described in legend to Figure 2a. *$P<0.001$; **$p<0.05$.

To further evaluate a possible association between disease manifestation and anti-self antibody production in naked DNA vaccinated rats the kinetics of anti-self antibody was carefully evaluated. Rats have been subjected to MCP-1, MIP-1α MIP-1β or RANTES naked DNA vaccines and then immunized with p68-86/CFA, as described under Figure 2a. At different time points (0, 3, 5, 7, 10, 12, 21, 30 and 40 days after EAE induction) generation of anti-self antibody was determined (Figures 5a-d). Each antibody titer profoundly increased within five to seven days of MBP p68-86/CFA immunization ($p<0.001$ compared to the day 0). The increase,

was simultaneous with the accelerated transcription of each chemokine mRNA at the site of inflammation (Figures 1a-d), suggesting that generation of each gene product at the site of inflammation elicits the production of anti-self antibodies. Each antibody titer peaked after the onset of disease (day 10-12) and returned to background within 40 days. Our data therefore clearly show that transcriptional up-regulation of individual chemokines in the CNS can provide protection from disease progression.

Finally, the competence of C-C chemokine naked DNA vaccines to render long lasting protective immunity against EAE was evaluated. Rats were subjected to three weakly injections of C-C chemokine naked DNA vaccines as described above (first experiment, Figure 2a). Two months after last vaccine was administered EAE was actively induced. Rats immunized with either MIP-1α or MCP-1 DNA vaccines were highly protected against EAE (incidence of 0/4 for each treatment, compared with 4/4 with a maximal score of 1.25±0.28 in either control and pcDNA treated rats, $p<0.001$ for each comparison). MIP-1β naked DNA vaccination, however, aggravated the disease (incidence 4/4 with a maximal score of 2.5±0.33, $p<0.013$ for each comparison).

Thus, MIP-1α and MCP-1 DNA vaccines generate long lasting protective immunity against autologous cytokines when such a response is needed.

Dynamics of transcription TNF-α mRNAs in the inflamed brain: Rats injected with L68-86 developed transferred EAE that persisted for 5-6 days (Figure 8a). Before adoptive transfer of disease (day 0), and at various time points: before the onset of disease (day 3), at the day of onset (day 5), the peak (day 7), following recovery (day 10), and 10 days after recovery (day 20) midbrain-brain stem samples were obtained from six different rats at each time point. From each sample mRNA was isolated and subjected to RT-PCR analysis using specific oligonucleotide primers. Each

amplification was calibrated to β-actin and verified by Southern Blotting analysis. This enabled semi-quantitative analysis of the dynamics of mRNA transcription of TNF-α at the site of inflammation. Figure 8b shows representative results from each time point of the experiment. Transcription of TNF-α in EAE brains was apparent at the onset of disease (day 5), peaked with at the time when clinical disease attained it maximal severity (day 7), and gradually regressed following recovery. Notable transcription could, Nevertheless, still be observed even ten days after recovery (Figure 8b).

Rats with developing active disease (Figure 8c) manifested similar mRNA transcription characteristics as those with developing transferred disease. That is, an apparent transcription of mRNA encoding TNF-α at the CNS just before the onset of disease (day 8) with a substantial augmentation at the time when clinical disease attained it maximal severity (day 13), and marked regression following recovery (Figure 8d).

Transcriptional induction of natural immune response to TNF-α in EAE rats: The development of anti-self immunity to TNF-α in EAE rats was evaluated. Just before active induction of disease (day 0) and when EAE attained its maximal severity (day 13, Figures 8a-b) blood samples were analyzed for the production of antibodies against self TNF-α. Rats, with developing EAE display a significantly increased TNF-α specific antibody titer as compared to rats immunized in hind foot pads with CFA alone (Figure 9, d Vs. b, log2 antibody titer of 11±0.85 Vs. 7±0.66, respectively, $p<0.05$).

These results are remarkable since both groups exhibited an extensive local inflammatory process at the site of CFA immunization (hind foot pads), with a massive local transcription of TNF-α mRNA (data not shown). Nevertheless, only rats with developing EAE manifested an apparent transcription of mRNA encoding TNF-α at the CNS that substantially increased at the time when clinical disease attained it maximal

severity (Figure 8d). In contrast, rats immunized with CFA alone did not exhibit a notable transcription of mRNA encoding TNF-α at the CNS of all detected brain samples at various time points (0, 8, 13 and 21 days after CFA administration, data not shown). Thus, only the transcription of the inflammatory cytokine TNF-α at an privileged autoimmune site (CNS) enabled the triggering of an anti-self response against this pro-inflammatory cytokine. This response was, however, not sufficient to prevent the development of an autoimmune condition (6/6 sick rats, Figure 10).

Prevention of EAE using TNF-α naked DNA vaccines: A PCR products of rat TNF-α, obtained as described above, was ligated into a pcDNA3 eukaryotic expression vector and used as constructs for naked DNA vaccination (Figure 10). Rats were subjected to three weakly injections of the above construct. Control rats were either injected with the pcDNA3 vector alone, or with PBS. Two months after the last immunization all rats were immunized with p68-86/CFA to induce active EAE. All control, i.e., PBS immunized and pcDNA3 vaccinated, rats developed active disease that persisted for 5-6 days (Figure 10, 6/6 in each group with a maximum clinical score 3±0.28 in control, 2.83±0.18 in pcDNA3 immunized rats). In contrast, rats injected the TNF-α naked DNA vaccine were resistant to EAE (incidence of 2/6 with a maximum clinical score of 0.33±0.2, $p<0.003$ for each the treatment of TNF-α DNA naked DNA vaccine compared with either control or pcDNA3 treatments). Thus, the subsequent *in vivo* immune response to TNF-α naked DNA vaccine prevented EAE.

Naked DNA encoding TNF-α augments transcriptionally regulated protective immunity: The development of anti-self protective immunity in DNA vaccinated rats was evaluated. When active EAE attained its maximal severity (day 13) blood samples of representative rats from the experiment described under Figure 10 were analyzed for the production of antibodies against TNF-α (Figure 9, d-e and h), and for the kinetics of antibody

production (Figure 11). The notable anti-self antibody titer against TNF-α produced in EAE rats, without DNA vaccination (Figure 9, d Vs. b, log2 antibody titer of 11±0.85 Vs. 7±0, respectively, $p<0.05$), which was not sufficient to prevent the development of disease, profoundly augmented in naked DNA vaccinated rats (Figure 9, h Vs. d and e, log2 antibody titer of 28±1.88 in rats vaccinated with the TNF-α construct Vs. 8±0.8 in EAE rats vaccinated with pcDNA3 alone and 11±0.85 in control EAE rats, respectively, $p<0.0001$ for the comparison of h with either d or e).

At different time points: 0, 8, 13, and 21 days after active EAE induction, the kinetics of anti-self TNF-α antibody production was determined (Figure 11). TNF-α specific antibody titer profoundly increased within eight days of MBP p68-86/CFA immunization (log2 antibody titer of 26±1.88 on day 8 Vs. 7±1 on day 0, $p<0.001$). The increase, was simultaneous with the accelerated transcription of TNF-α mRNA at the site of inflammation (Figures 8a-f). At this time a highly significant difference could be observed between the production of TNF-α specific antibody titer in TNF-α DNA vaccinated rats that were immunized with p68-86/CFA to those immunized with CFA alone (log2 antibody titer of 26±1.88 Vs. 13±0.6, $p<0.001$). Thus, administration of naked DNA encoding TNF-α augments transcriptionally regulated generation anti-self antibodies against a proinflammatory cytokine that are involved in the induction and progression of the autoimmune condition.

TNF-α specific anti-self antibodies from naked DNA vaccinated rats transfer EAE resistance: Since DNA vaccination elicits both cellular and humoral responses against products of a given construct it is difficult to know which of these responses contributed more to the development of EAE resistance in TNF-α DNA vaccinated rats. To evaluate the possible contribution of anti-self antibodies to the development of EAE resistance twelve-thirteen days after active induction of EAE, when production of anti-self antibodies in naked DNA vaccinated rats attained at its maximal titer

(Figure 11), antibodies were purified (IgG fraction, protein G purification) and evaluated for their competence to provide subsequent protection from severe EAE (Figure 12). Four days before the onset of active EAE, rats were daily challenged (days 6-13) with 100 μg of each of antibodies, or with antibodies from rats that were vaccinated with either pcDNA3-TNF-α construct, or with pcDNA3 alone. Repeated administration of antibodies from TNF-α DNA vaccinated rats provided substantial protection from disease progression (Mean maximal score of 1±0.2 in rats treated with purified antibodies from TNF-α DNA vaccinated donors Vs. 2.66±03 and 2.83±0816 in rats treated with purified antibodies from PBS or pcDNA3 treated rats, respectively, $p<0.001$ for each compression).

Taken together these results show that administration of naked DNA encoding TNF-α augments transcriptionally regulated generation anti-self antibodies capable of blocking the development of an experimental autoimmune disease of the CNS and thus providing a tool by which the immune system is encouraged to elicit anti-self protective immunity to restrain its own harmful reactivity only when such a response is needed.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

LIST OF REFERENCES CITED

1. Alvord, E. C. J., M. W. Kies, and A. J. Suckling. 1984. Experimental Allergic Encephalomylitis: A useful Model for Multiple Sclerosis. In *Progress in clinical and biological research., vol. 146.* E. C. J. Alvord, M. W. Kies, and A. J. Suckling, eds. Allen R. Liss, New York, p. 1-537.
2. MacFarlin, D., and H. MacFarland. 1983. Multiple Sclerosis. *N. Engl.J.Med. 307:1183-1188.*
3. Karin, N., F. Szafer, D. Mitchell, D. P. Gold, and L. Steinman. 1993. Selective and nonselective stages in homing of T lymphocytes to the central nervous system during experimental allergic encephalomyelitis. *J Immunol 150:4116-24.*
4. Yednock, T. A., C. Cannon, L. C. Fritz, M. F. Sanchez, L. Steinman, and N. Karin. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature 356:63-6.*
5. Brocke, S., K. Gijbels, M. Allegretta, I. Ferber, C. Piercy, T. Blankenstein, R. Martin, U. Utz, N. Karin, D. Mitchell, and *at al.* 1996. Treatment of experimental encephalomyelitis with a peptide analog of myelin basic protein. *Nature 379:343-6.*
6. Schmied, M., H. Breitschopf, R. Gold, H. Zischler, G. Rothe, H. Wekerle, and H. Lassmann. 1993. Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis; Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Americam Journal of Pathology 143:446-451.*
7. Karin, N., J. D. Mitchell, S. Brocke, N. Ling, and L. Steinman. 1994. Reversal of experimental autoimmune encephalomyelitis by as soluble peptide variant of a myelin basic protein epitope: T cell

receptor antagonism and reduction of IFN-g and TNF-α production. *J. Exp. Med. 180:2227-2237.*

8. Xiang, Z., and H. C. Ertl. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity 2:129-35.*
9. Irvine, K. R., J. B. Rao, S. A. Rosenberg, and N. P. Restifo. 1996. Cytokine enhancement of DNA immunization leads to effective treatment of established pulmonary metastases. *Journal of Immunology 156:238-45.*
10. Ulmer, J. B., J. C. Sadoff, and M. A. Liu. 1996. DNA vaccines. *Current opinion in immunology 8:531-536.*
11. Barry, M. A., W. C. Lai, and S. A. Johnston. 1995. Protection against mycoplasma infection using expression-library immunization. *Nature 377:632-5.*
12. Sedegah, M., R. Hedstrom, P. Hobart, and S. L. Hoffman. 1994. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc Natl Acad Sci U S A 91:9866-70.*
13. Tang, D. C., M. DeVit, and S. A. Johnston. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature 356:152-4.*
14. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, and *at al.* 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein [see comments]. *Science 259:1745-9.*
15. Sato, Y., M. Roman, H. Tighe, D. Lee, M. Corr, M. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science.*

16. Singh, R. R., V. Kumar, F. M. Ebling, S. Southwood, A. Sette, E. E. Sercarz, and B. H. Hahn. 1995. T cell determinants from autoantibodies to DNA can upregulate autoimmunity in murine systemic lupus erythematosus. *Journal of Experimental Medicine 181:2017-27.*

17. Waisman, A., P. J. Ruiz, D. L. Hirschberg, A. Gelman, J. R. Oksenberg, S. Brocke, F. Mor, I. R. Cohen, and L. Steinman. 1996. Suppressive vaccination with DNA encoding a variable region gene of the T-cell receptor prevents autoimmune encephalomyelitis and activates Th2 immunity. *Nature Medicine 2:899-905.*

18. Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik, and T. J. Schall. 1997. A new class of membrane-bound chemokine with a CX3C motif. *Nature 385:640-4.*

19. Pan, Y., C. Lloyd, H. Zhou, S. Dolich, J. Deeds, J. A. Gonzalo, J. Vath, M. Gosselin, J. Ma, B. Dussault, E. Woolf, G. Alperin, J. Culpepper, J. C. Gutierrez-Ramos, and D. Gearing. 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature 387:611-7.*

20. Ben-Baruch, A., D. F. Michiel, and J. J. Oppenheim. 1995. Signals and receptors involved in recruitment of inflammatory cells. *J Biol Chem 270:11703-6.*

21. Ponath, P. D., S. Qin, D. J. Ringler, I. Clark-Lewis, J. Wang, N. Kassam, H. Smith, X. Shi, J. A. Gonzalo, W. Newman, J. C. Gutierrez-Ramos, and C. R. Mackay. 1996. Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J Clin Invest 97:604-12.*

22. Carr, M. W., R. Alon, and T. A. Springer. 1996. The C-C chemokine MCP-1 differentially modulates the avidity of beta 1 and beta 2 integrins on T lymphocytes. *Immunity 4:179-87.*

23. Lloyd, A. R., J. J. Oppenheim, D. J. Kelvin, and D. D. Taub. 1996. Chemokines regulate T cell adherence to recombinant adhesion molecules and extracellular matrix proteins. *Journal of Immunology 156:932-8.*

24. Bacon, K. B., L. Flores-Romo, J. P. Aubry, T. N. Wells, and C. A. Power. 1994. Interleukin-8 and RANTES induce the adhesion of the human basophilic cell line KU-812 to human endothelial cell monolayers. *Immunology 82:473-81.*

25. Brown, Z., M. E. Gerritsen, W. W. Carley, R. M. Strieter, S. L. Kunkel, and J. Westwick. 1994. Chemokine gene expression and secretion by cytokine-activated human microvascular endothelial cells. Differential regulation of monocyte chemoattractant protein-1 and interleukin-8 in response to interferon-gamma. *American Journal of Pathology 145:913-21.*

26. Carlos, T. M., and J. M. Harlan. 1994. Leukocyte-endothelial adhesion molecules. *Blood 84:2068-101.*

27. Jutila, M. A. 1994. Role of changes in the vascular endothelium in chronic inflammation. *Clinical Transplantation 8:304-7.*

28. Kim, J. S., S. C. Gautam, M. Chopp, C. Zaloga, M. L. Jones, P. A. Ward, and K. M. Welch. 1995. Expression of monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 after focal cerebral ischemia in the rat. *Journal of Neuroimmunology 56:127-34.*

29. Lukacs, N. W., R. M. Strieter, V. Elner, H. L. Evanoff, M. D. Burdick, and S. L. Kunkel. 1995. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. *Blood 86:2767-73.*

30. Schall, T. J., K. Bacon, R. D. Camp, J. W. Kaspari, and D. V. Goeddel. 1993. Human macrophage inflammatory protein alpha (MIP-1 alpha) and MIP-1 beta chemokines attract distinct populations of lymphocytes. *Journal of Experimental Medicine 177:1821-6.*

31. Vaddi, K., and R. C. Newton. 1994. Regulation of monocyte integrin expression by beta-family chemokines. *Journal of Immunology 153:4721-32.*

32. Yu, X., and D. T. Graves. 1995. Fibroblasts, mononuclear phagocytes, and endothelial cells express monocyte chemoattractant protein-1 (MCP-1) in inflamed human gingiva. *Journal of Periodontology 66:80-8.*

33. Karpus, W. J., N. W. Lukacs, B. L. McRae, R. M. Strieter, S. L. Kunkel, and S. D. Miller. 1995. An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. *J Immunol 155:5003-10.*

34. Gong, J. H., L. G. Ratkay, J. D. Waterfield, and I. Clark-Lewis. 1997. An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. *J Exp Med 186:131-7.*

35. Berman, J. W., M. P. Guida, J. Warren, J. Amat, and C. F. Brosnan. 1996. Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in the rat. *J. Immunol. 156:3017-3023.*

36. Ben-Nun, A., H. Wekerle, and I. R. Cohen. 1981. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol 11:195-9.*

37. Lanir, N., P. S. Ciano, L. Van de Water, J. McDonagh, A. M. Dvorak, and H. F. Dvorak. 1988. Macrophage migration in fibrin gel matrices. II. Effects of clotting factor XIII, fibronectin, and glycosaminoglycan content on cell migration. *J Immunol 140:2340-9.*

38. Luo, Y., J. Laning, S. Devi, J. Mak, T. J. Schall, and M. E. Dorf. 1994. Biologic activities of the murine beta-chemokine TCA3. *J Immunol 153:4616-24.*

39. Raz, E., H. Tighe, Y. Sato, M. Corr, J. A. Dudler, M. Roman, S. L. Swain, H. L. Spiegelberg, and D. A. Carson. 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci U S A 93:5141-5.*

40. Godiska, R., D. Chantry, G. N. Dietsch, and P. W. Gray. 1995. Chemokine expression in murine experimental allergic encephalomyelitis. *J Neuroimmunol 58:167-76.*

41. Kim, J. J., M. L. Bagarazzi, N. Trivedi, Y. Hu, K. Kazahaya, D. M. Wilson, R. Ciccarelli, M. A. Chattergoon, K. Dang, S. Mahalingam, A. A. Chalian, M. G. Agadjanyan, J. D. Boyer, B. Wang, and D. B. Weiner. 1997. Engineering of in vivo immune responses to DNA immunization via codelivery of costimulatory molecule genes. *Nat Biotechnol 15:641-6.*

42. Kim, J. J., V. Ayyavoo, M. L. Bagarazzi, M. A. Chattergoon, K. Dang, B. Wang, J. D. Boyer, and D. B. Weiner. 1997. In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. *J Immunol 158:816-26.*

43. Fu, T. M., J. B. Ulmer, M. J. Caulfield, R. R. Deck, A. Friedman, S. Wang, X. Liu, J. J. Donnelly, and M. A. Liu. 1997. Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol Med 3:362-71.*

44. Carr, M. W., S. J. Roth, E. Luther, S. S. Rose, and T. A. Springer. 1994. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A 91:3652-6.*

45. Lloyd, C. M., A. W. Minto, M. E. Dorf, A. Proudfoot, T. N. Wells, D. J. Salant, and J. C. Gutierrez-Ramos. 1997. RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J Exp Med 185:1371-80.*

46. Uguccioni, M., M. D'Apuzzo, M. Loetscher, B. Dewald, and M. Baggiolini. 1995. Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1 alpha and MIP-1 beta on human monocytes. *Eur J Immunol 25:64-8.*

47. del Pozo, M. A., P. Sanchez-Mateos, M. Nieto, and F. Sanchez-Madrid. 1995. Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. *J Cell Biol 131:495-508.*

48. Lukacs, N. W., R. M. Strieter, V. M. Elner, H. L. Evanoff, M. Burdick, and S. L. Kunkel. 1994. Intercellular adhesion molecule-1 mediates the expression of monocyte-derived MIP-1 alpha during monocyte-endothelial cell interactions. *Blood 83:1174-8.*

49. Weber, C., R. Alon, B. Moser, and T. A. Springer. 1996. Sequential regulation of alpha 4 beta 1 and alpha 5 beta 1 integrin avidity by CC chemokines in monocytes: implications for transendothelial chemotaxis. *J Cell Biol 134:1063-73.*

50. Riethmuller, G., E. P. Rieber, S. Kiefersauer, J. Prinz, P. van der Lubbe, B. Meiser, F. Breedveld, J. Eisenburg, K. Kruger, K. Deusch, and *at al.* 1992. From antilymphocyte serum to therapeutic monoclonal antibodies: first experiences with a chimeric CD4 antibody in the treatment of autoimmune disease. *Immunol Rev 129:81-104.*

51. Green, L. L., M. C. Hardy, C. E. Maynard-Currie, H. Tsuda, D. M. Louie, M. J. Mendez, H. Abderrahim, M. Noguchi, D. H. Smith, Y.

Zeng, and *at al.* 1994. Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. *Nat Genet 7:13-21.*

52. Glabinski, A. R., M. Tani, R. M. Strieter, V. K. Tuohy, and R. M. Ransohoff. 1997. Synchronous synthesis of alpha- and beta-chemokines by cells of diverse lineage in the central nervous system of mice with relapses of chronic experimental autoimmune encephalomyelitis. *Am J Pathol 150:617-30.*
53. Issazadeh, S., A. Ljungdahl, B. Hojeberg, M. Mustafa, and T. Olsson. 1995. Cytokine production in the central nervous system of Lewis rats with experimental autoimmune encephalomyelitis: dynamics of mRNA expression for interleukin-10, interleukin-12, cytolysin, tumor necrosis factor alpha and tumor necrosis factor beta. *J Neuroimmunol 61:205-12.*
54. Kennedy, M. K., D. S. Torrance, K. S. Picha, and K. M. Mohler. 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J Immunol 149:2496-505.*
55. Villarroya, H., Y. Marie, J. C. Ouallet, F. Le Saux, J. L. chelingerian, and N. Baumann. 1997. Expression of TNF alpha in central neurons of Lewis rat spinal cord after EAE induction. *J Neurosci Res 49:592-9.*
56. Liu, J., M. W. Marino, G. Wong, D. Grail, A. Dunn, J. Bettadapura, A. J. Slavin, L. Old, and C. C. Bernard. 1998. TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. *Nature Medicine 4:78-83.*
57. Kuroda, Y., and Y. Shimamoto. 1991. Human tumor necrosis factor-alpha augments experimental allergic encephalomyelitis in rats. *J Neuroimmunol 34:159-64.*

58. Powell, M. B., D. Mitchell, J. Lederman, J. Buckmeier, S. S. Zamvil, M. Graham, N. H. Ruddle, and L. Steinman. 1990. Lymphotoxin and tumor necrosis factor-alpha production by myelin basic protein-specific T cell clones correlates with encephalitogenicity. *Int Immunol 2:539-44.*

59. Ruddle, N. H., C. M. Bergman, K. M. McGrath, E. G. Lingenheld, M. L. Grunnet, S. J. Padula, and R. B. Clark. 1990. An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J Exp Med 172:1193-200.*

60. Selmaj, K., C. S. Raine, and A. H. Cross. 1991. Anti-tumor necrosis factor therapy abrogates autoimmune demyelination. *Ann Neurol 30:694-700.*

61. Thornhill, M. H., S. M. Wellicome, D. L. Mahiouz, J. S. Lanchbury, A. U. Kyan, and D. O. Haskard. 1991. Tumor necrosis factor combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. *J Immunol 146:592-8.*

62. Korner, H., F. A. Lemckert, G. Chaudhri, S. Etteldorf, and J. D. Sedgwick. 1997. Tumor necrosis factor blockade in actively induced experimental autoimmune encephalomyelitis prevents clinical disease despite activated T cell infiltration to the central nervous system. *Eur J Immunol 27:1973-81.*

63. Korner, H., D. S. Riminton, D. H. Strickland, F. A. Lemckert, J. D. Pollard, and J. D. Sedgwick. 1997. Critical points of tumor necrosis factor action in central nervous system autoimmune inflammation defined by gene targeting. *J Exp Med 186:1585-90.*

64. Suen, W. E., C. M. Bergman, P. Hjelmstrom, and N. H. Ruddle. 1997. A critical role for lymphotoxin in experimental allergic encephalomyelitis. *J Exp Med 186:1233-40.*

65. Taupin, V., T. Renno, L. Bourbonniere, A. C. Peterson, M. Rodriguez, and T. Owens. 1997. Increased severity of experimental autoimmune encephalomyelitis, chronic macrophage/microglial reactivity, and demyelination in transgenic mice producing tumor necrosis factor-alpha in the central nervous system. *Eur J Immunol 27:905-13.*

66. Brenner, T., S. Brocke, F. Szafer, R. A. Sobel, J. F. Parkinson, D. H. Perez, and L. Steinman. 1997. Inhibition of nitric oxide synthase for treatment of experimental autoimmune encephalomyelitis. *J Immunol 158:2940-6.*

67. Frei, K., H. P. Eugster, M. Bopst, C. S. Constantinescu, E. Lavi, and A. Fontana. 1997. Tumor necrosis factor alpha and lymphotoxin alpha are not required for induction of acute experimental autoimmune encephalomyelitis. *J Exp Med 185:2177-82.*

68. Pan, W., W. A. Banks, M. K. Kennedy, E. G. Gutierrez, and A. J. Kastin. 1996. Differential permeability of the BBB in acute EAE: enhanced transport of TNT-alpha. *Am J Physiol 271:E636-42.*

69. Becher, B., V. Dodelet, V. Fedorowicz, and J. P. Antel. 1996. Soluble tumor necrosis factor receptor inhibits interleukin 12 production by stimulated human adult microglial cells in vitro. *J Clin Invest 98:1539-43.*

70. Leonard, J. P., K. E. Waldburger, and S. J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J Exp Med 181:381-6.*

71. Selmaj, K., W. Papierz, A. Glabinski, and T. Kohno. 1995. Prevention of chronic relapsing experimental autoimmune encephalomyelitis by soluble tumor necrosis factor receptor I. *J Neuroimmunol 56:135-41.*

72. Pette, M., K. Fujita, B. Kitze, J. N. Whitaker, E. Albert, L. Kappos, and H. Wekerle. 1990. Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. *Neurology 40:1770-6.*

73. Chen, Y., V. K. Kuchroo, J. Inobe, D. Hafler, and H. L. Weiner. 1994. Regulatory T-cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. *Science 265:1237-1240.*

74. Rapoport, M. J., A. Jaramillo, D. Zipris, A. Lazarus, D. V. Serreze, E. H. Leiter, P. Cyopick, J. S. Danska, and T. L. Delovitch. 1993. Interleukin-4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *J. Exp. Med. 178:87-99.*

75. Friedman, A., and H. L. Weiner. 1994. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci U S A 91:6688-6692.*

76. Khoury, S. J., W. W. Hancock, and H. L. Weiner. 1992. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor beta, interleukin 4, and prostaglandin E expression in the brain. *J Exp Med 176:1355-64.*

77. Cash, E., A. Minty, P. Ferrara, D. Caput, D. Fradelizi, and O. Rott. 1994. Macrophage-inactivating IL-13 suppresses experimental autoimmune encephalomyelitis in rats. *J Immunol 153:4258-67.*

78. Saoudi, A., J. Kuhn, K. Huygen, K. Y. de, T. Velu, M. Goldman, P. Druet, and B. Bellon. 1993. TH2 activated cells prevent experimental autoimmune uveoretinitis, a TH1-dependent autoimmune disease. *Eur J Immunol 23:3096-103.*

79. Liblau, R. S., S. M. Singer, and H. O. McDevitt. 1994. Th1 and Th2 CD4+ T-Cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today in press.*

80. Racke, M. K., J. S. Dhib, B. Cannella, P. S. Albert, C. S. Raine, and D. E. McFarlin. 1991. Prevention and treatment of chronic relapsing

experimental allergic encephalomyelitis by transforming growth factor-beta 1. *J Immunol 146:3012-7.*

81. Racke, M. K., A. Bonomo, D. E. Scott, B. Cannella, A. Levine, C. S. Raine, E. M. Shevach, and M. Rocken. 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J Exp Med 180:1961-6.*
82. Segal, B. M., B. K. Dwyer, and E. M. Shevach. 1998. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. *J Exp Med 187:537-46.*
83. Steinman, L. 1995. Escape from "horror autotoxicus": pathogenesis and treatment of autoimmune disease. *Cell 80:7-10.*
84. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol 12:991-1045.*
85. Janeway, C. A., Jr. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today 13:11-6.*
86. Cyster, J. G., S. B. Hartley, and C. C. Goodnow. 1994. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire [see comments]. *Nature 371:389-95.*

WHAT IS CLAIMED IS:

1. A method for treating a mammal for inducing protective immunity against an autoimmune disease, the method comprising the step of administering to said mammal a therapeutic composition including a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, said nucleic acid sequence being operatively linked to one or more transcription control sequences.

2. The method of claim 1, wherein said cytokine is a chemokine or tumor necrosis factor alpha.

3. The method of claim 2, wherein said chemokine is a C-C chemokine.

4. The method of claim 3, wherein said C-C chemokine is selected from the group consisting of macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES).

5. The method of claim 1, wherein said transcription control sequences are selected from the group consisting of RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

6. The method of claim 1, wherein said recombinant construct is an eukaryotic expression vector.

7. The method of claim 1, wherein said recombinant construct is selected from the group consisting of pcDNA3, pcDNA3:1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives..

8. The method of claim 1, wherein said therapeutic composition is administered to the mammal parenterally.

9. The method of claim 1, wherein said mammal is a human.

10. The method of claim 1, wherein said mammal is selected from the group consisting of humans, dogs, cats, sheep, cattle, horses and pigs.

11. The method of claim 1, wherein said autoimmune disease is multiple sclerosis.

12. The method of claim 11, wherein said cytokine is a chemokine or tumor necrosis factor alpha.

13. The method of claim 12, wherein said chemokine is a C-C chemokine.

14. The method of claim 13, wherein said C-C chemokine is selected from the group consisting of macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES).

15. The method of claim 11, wherein said transcription control sequences are selected from the group consisting of RSV control sequences,

CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

16. The method of claim 11, wherein said recombinant construct is an eukaryotic expression vector.

17. The method of claim 11, wherein said recombinant construct is selected from the group consisting of pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives..

18. The method of claim 11, wherein said therapeutic composition is administered to the mammal parenterally.

19. The method of claim 11, wherein said mammal is a human.

20. The method of claim 11, wherein said mammal is selected from the group consisting of humans, dogs, cats, sheep, cattle, horses and pigs.

21. A method for treating a mammal for inducing protective immunity against an autoimmune disease, the method comprising the steps of:

(a) removing cells of said mammal;

(b) transducing said cells in vitro with a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, said nucleic acid sequence being operatively linked to one or more transcription control sequences; and

(c) reintroducing said transduced cells to said mammal.

22. The method of claim 21, wherein said cytokine is a chemokine or tumor necrosis factor alpha.

23. The method of claim 22, wherein said chemokine is a C-C chemokine.

24. The method of claim 23, wherein said C-C chemokine is selected from the group consisting of macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES).

25. The method of claim 21, wherein said transcription control sequences are selected from the group consisting of RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

26. The method of claim 21, wherein said recombinant construct is an eukaryotic expression vector.

27. The method of claim 21, wherein said recombinant construct is selected from the group consisting of pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives.

28. The method of claim 21, wherein said transduced cells are reintroduced to the mammal parenterally.

29. The method of claim 21, wherein said mammal is a human.

30. The method of claim 21, wherein said mammal is selected from the group consisting of humans, dogs, cats, sheep, cattle, horses and pigs.

31. The method of claim 21, wherein said autoimmune disease is multiple sclerosis.

32. The method of claim 31, wherein said cytokine is a chemokine or tumor necrosis factor alpha.

33. The method of claim 32, wherein said chemokine is a C-C chemokine.

34. The method of claim 33, wherein said C-C chemokine is selected from the group consisting of macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES).

35. The method of claim 31, wherein said transcription control sequences are selected from the group consisting of RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

36. The method of claim 31, wherein said recombinant construct is an eukaryotic expression vector.

37. The method of claim 31, wherein said recombinant construct is selected from the group consisting of pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives.

38. The method of claim 31, wherein said transduced cells are reintroduced to the mammal parenterally.

39. The method of claim 31, wherein said mammal is a human.

40. The method of claim 31, wherein said mammal is selected from the group consisting of humans, dogs, cats, sheep, cattle, horses and pigs.

41. A pharmaceutical composition comprising:

(a) a recombinant construct including an isolated nucleic acid sequence encoding a cytokine, said nucleic acid sequence being operatively linked to one or more transcription control sequences; and

(b) a pharmaceutically acceptable carrier.

42. The pharmaceutical composition of claim 41, wherein said pharmaceutically acceptable carrier is selected from the group consisting of an aqueous physiologically balanced solution, an artificial lipid-containing substrate, a natural lipid-containing substrate, an oil, an ester, a glycol, a virus and metal particles.

43. The pharmaceutical composition of claim 41, wherein said pharmaceutically acceptable carrier comprises a delivery vehicle that delivers said nucleic acid sequences to said mammal.

44. The pharmaceutical composition of claim 43, wherein said delivery vehicle is selected from the group consisting of liposomes, micelles, and cells.

45. The pharmaceutical composition of claim 41, wherein said composition is useful for treating an autoimmune disease.

46. The pharmaceutical composition of claim 45, wherein said autoimmune disease is multiple sclerosis.

47. The pharmaceutical composition of claim 41, wherein said cytokine is a chemokine or tumor necrosis factor alpha.

48. The pharmaceutical composition of claim 47, wherein said chemokine is a C-C chemokine.

49. The pharmaceutical composition of claim 48, wherein said C-C chemokine is selected from the group consisting of macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES).

50. The pharmaceutical composition of claim 41, wherein said transcription control sequences are selected from the group consisting of RSV control sequences, CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

51. The pharmaceutical composition of claim 41, wherein said recombinant construct is an eukaryotic expression vector.

52. The pharmaceutical composition of claim 41, wherein said recombinant construct is selected from the group consisting of pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives.

53. The pharmaceutical composition of claim 41, wherein said composition is suitable for parenteral administration to a human.

54. An antibody raised against a cytokine expressed by cells transduced with a recombinant construct including an isolated nucleic acid sequence encoding said cytokine, said nucleic acid sequence being operatively linked to one or more transcription control sequences.

55. The antibody of claim 54, wherein said cytokine is a chemokine or tumor necrosis factor alpha.

56. The antibody of claim 55, wherein said chemokine is a C-C chemokine.

57. The antibody of claim 56, wherein said C-C chemokine is selected from the group consisting of macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β) and regulation on activation normal T expressed and secreted (RANTES).

58. The antibody of claim 54, wherein said transcription control sequences are selected from the group consisting of RSV control sequences,

CMV control sequences, retroviral LTR sequences, SV-40 control sequences and β-actin control sequences.

59. The antibody of claim 54, wherein said recombinant construct is an eukaryotic expression vector.

60. The antibody of claim 54, wherein said recombinant construct is selected from the group consisting of pcDNA3, pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pCI, pBK-RSV, pBK-CMV, pTRES and their derivatives.

FIG. 1A

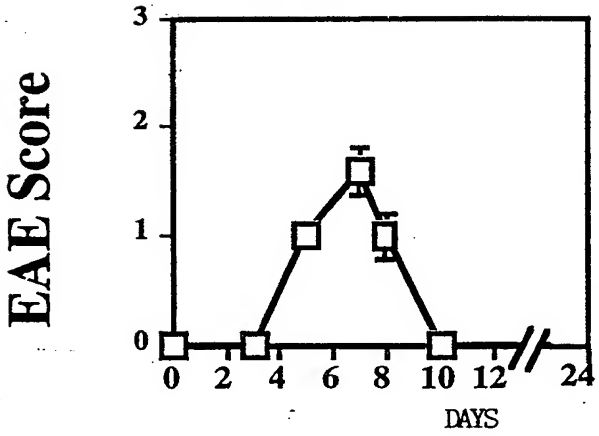


FIG. 1C

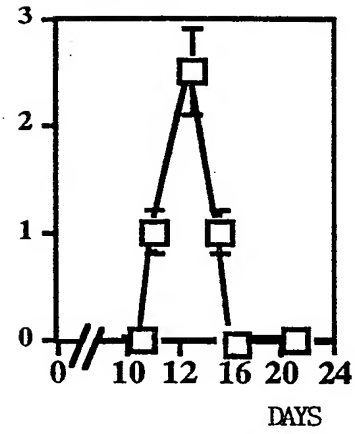


FIG. 1B

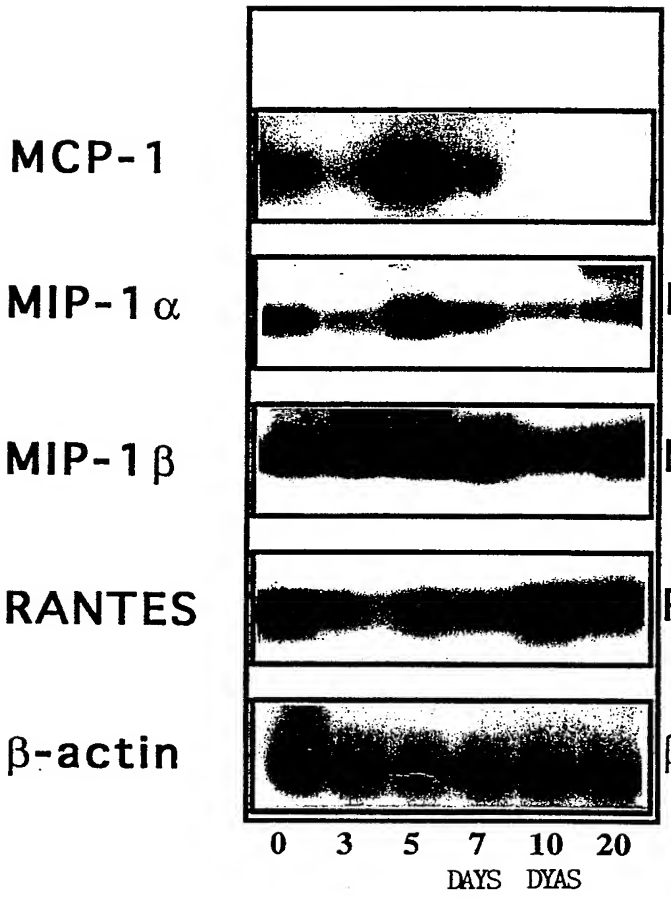


FIG. 1D

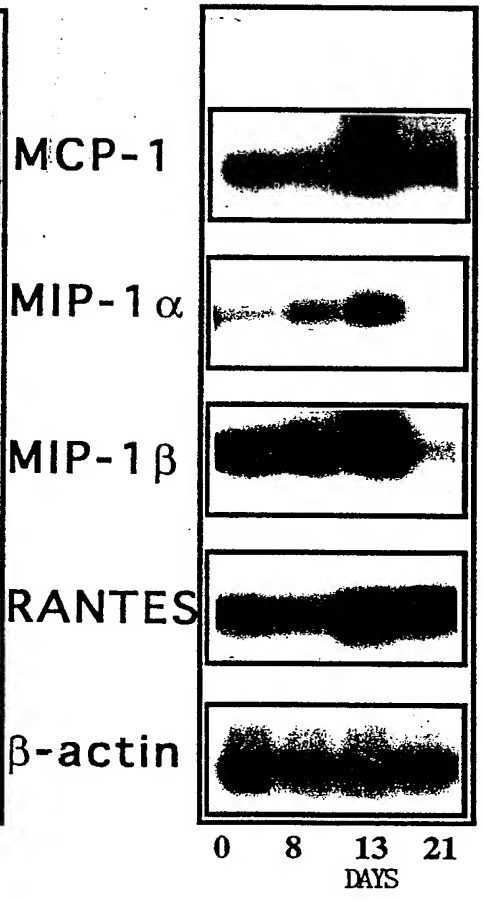
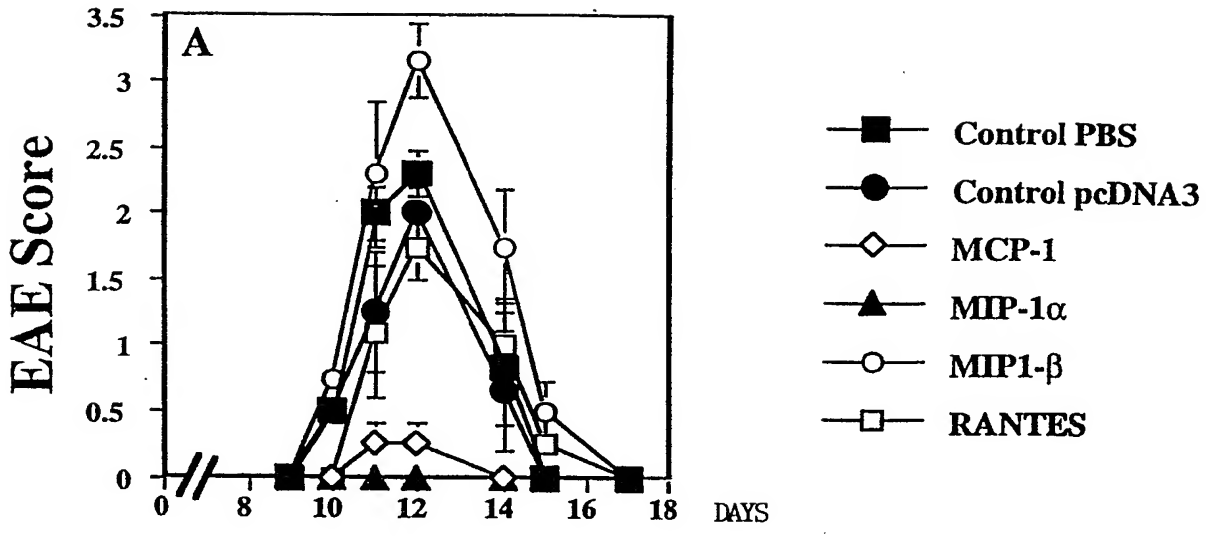


FIG. 2A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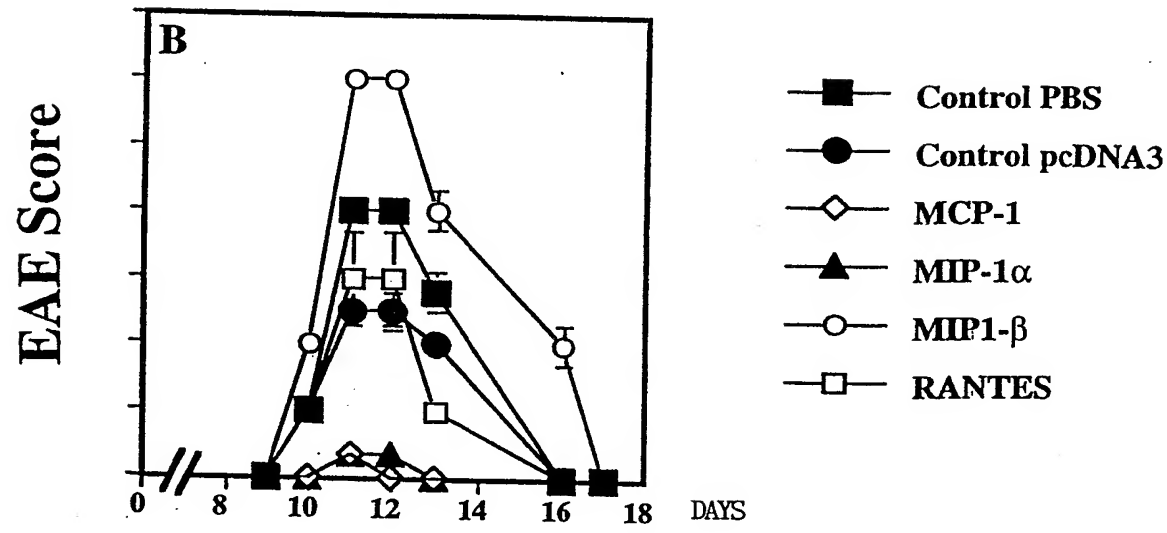
EAE Score
3.5
3
2.5
2
1.5
1
0.5
0
0
8
10
12
14
16
18
DAYS
Control PBS
Control pcDNA3
MCP-1
MIP-1α
MIP1-β
RANTES

FIG. 2B


B
EAE Score
0
8
10
12
14
16
18
DAYS
Control PBS
Control pcDNA3
MCP-1
MIP-1α
MIP1-β
RANTES

No EAE

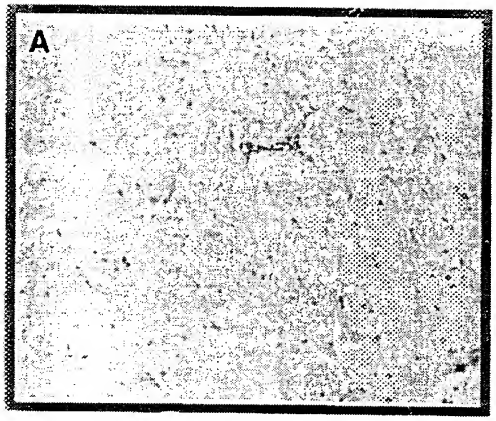


FIG. 3A

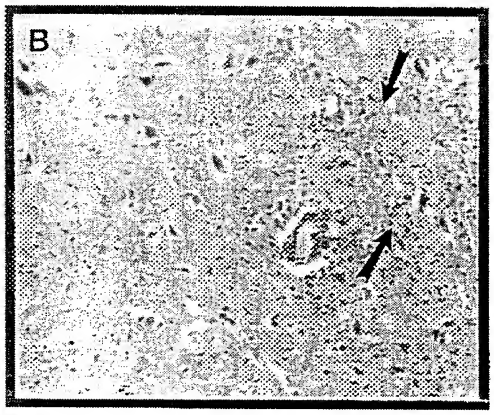


FIG. 3B

pcDNA3 alone

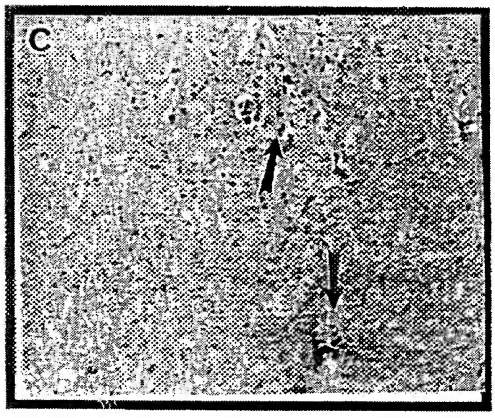


FIG. 3c

pcDNA3 /MCP-1

FIG. 3D

pcDNA3 /MIP-1α

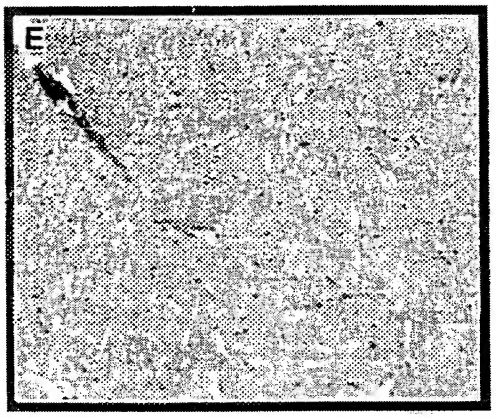


FIG. 3E

pcDNA3 /MIP-1β

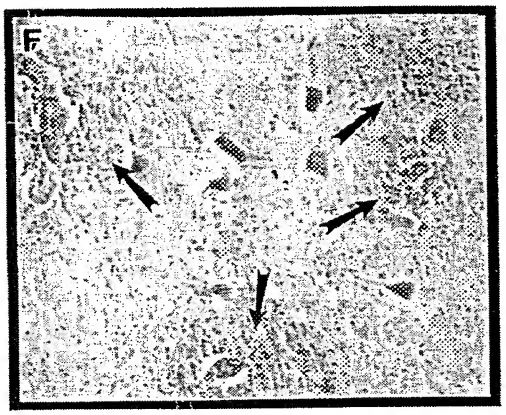


FIG. 3F

pcDNA3 /RANTES

FIG. 3G

FIG. 4A

DNA vaccination	EAE induction
-	-
pcDNA3	-
-	+
pcDNA3	+
pcDNA3-MCP-1	-
pcDNA3-MCP-1	CFA alone
pcDNA3-MCP-1	+

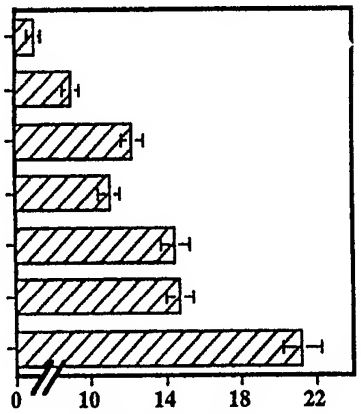


FIG. 4B

DNA vaccination	EAE induction
-	-
pcDNA3	-
-	+
pcDNA3	+
pcDNA3-MIP-1α	-
pcDNA3-MIP-1α	CFA alone
pcDNA3-MIP-1α	+

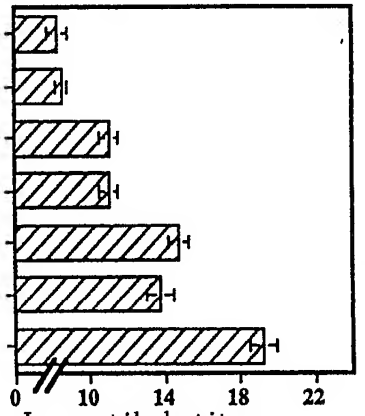


FIG. 4C

DNA vaccination	EAE induction
-	-
pcDNA3	-
-	+
pcDNA3	+
pcDNA3-MIP-1β	-
pcDNA3-MIP-1β	CFA alone
pcDNA3-MIP-1β	+

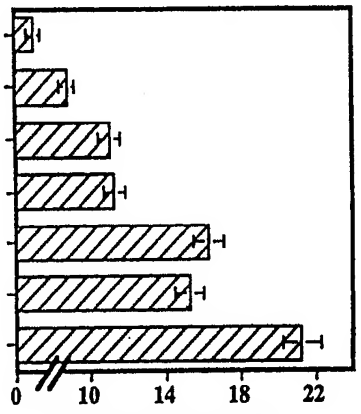
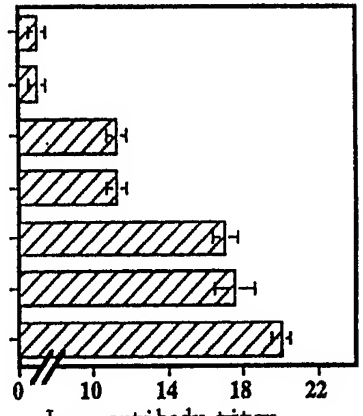


FIG. 4D

DNA vaccination	EAE induction
-	-
pcDNA3	-
-	+
pcDNA3	+
pcDNA3-RANTES	-
pcDNA3-RANTES	CFA alone
pcDNA3-RANTES	+



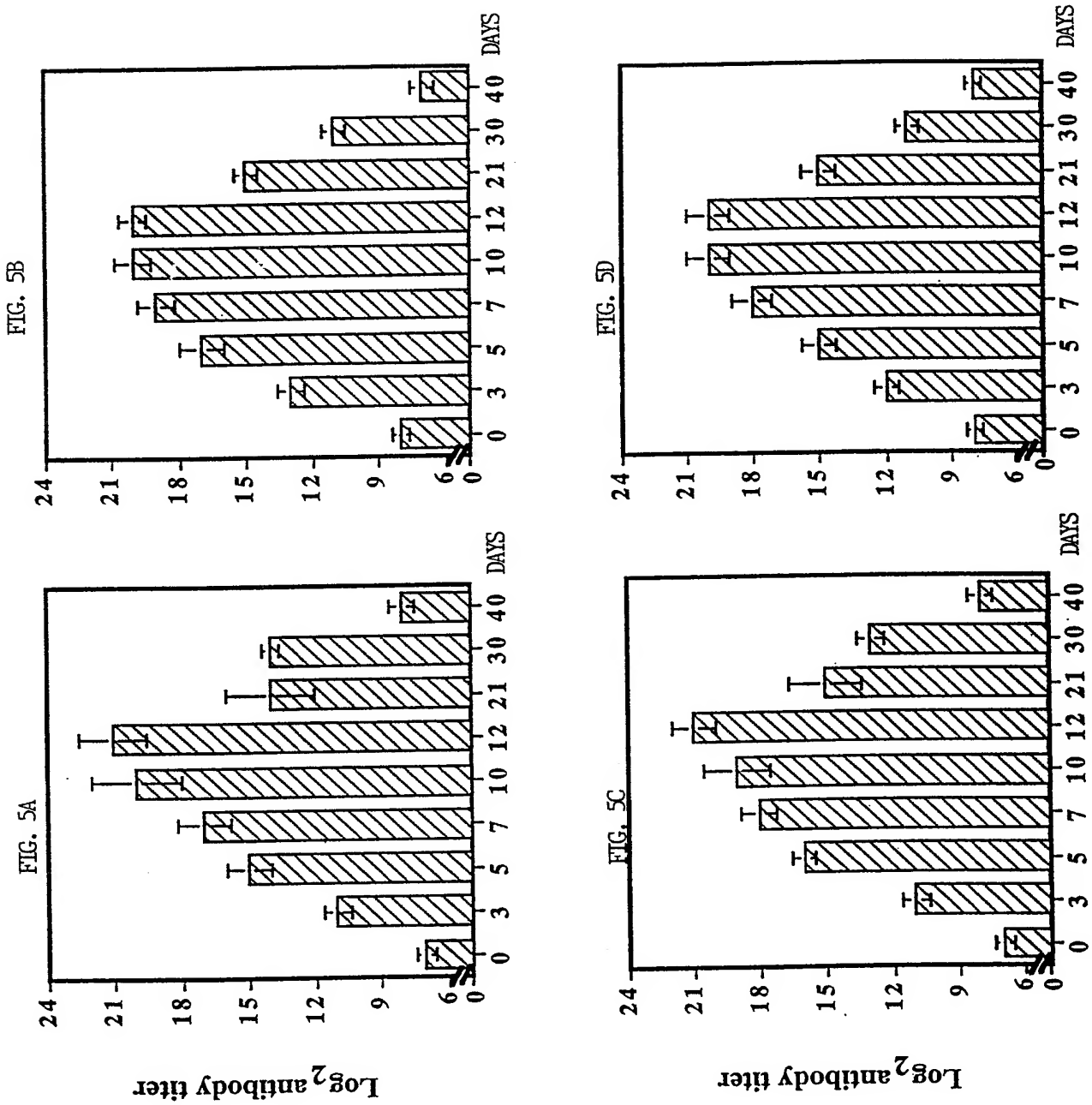


FIG. 5A
FIG. 5B
FIG. 5C
FIG. 5D
Log2 antibody titer
DAYS
0 3 5 7 10 12 21 30 40
6 9 12 15 18 21 24

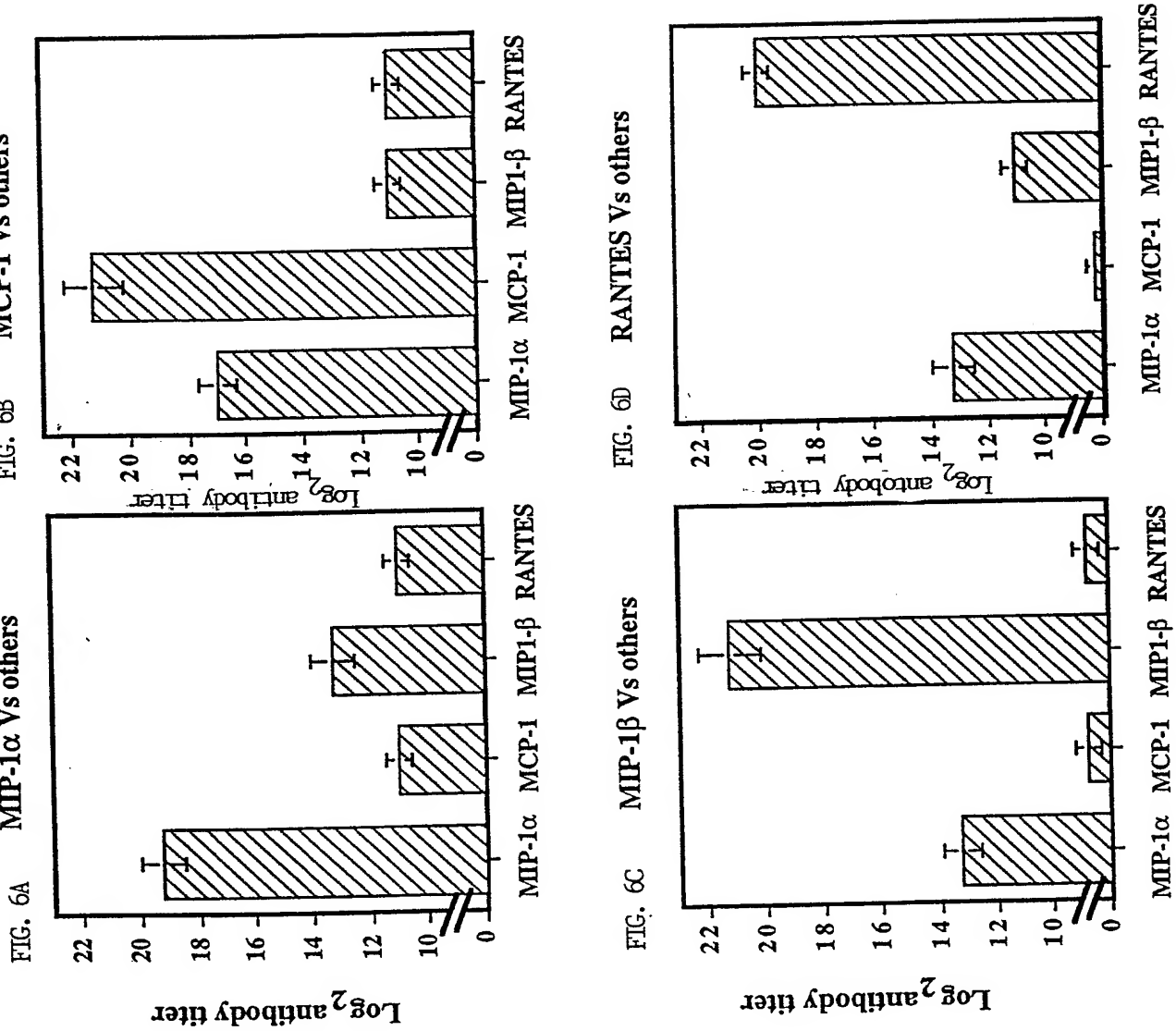


FIG. 6A
MIP-1α Vs others
FIG. 6B
MCP-1 Vs others
FIG. 6C
MIP-1β Vs others
FIG. 6D
RANTES Vs others
Log2 antibody titer
22
20
18
16
14
12
10
0
MIP-1α
MCP-1
MIP1-β
RANTES

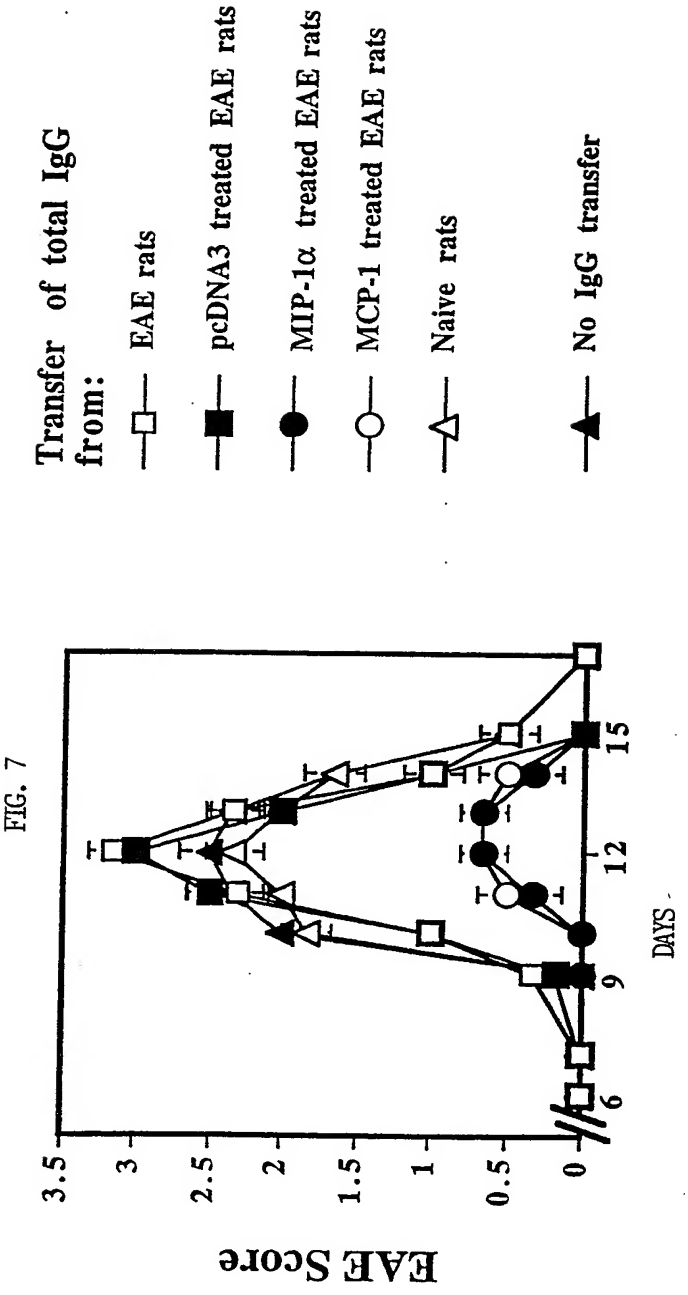

Transfer of total IgG from:
EAE rats
pcDNA3 treated EAE rats
MIP-1α treated EAE rats
MCP-1 treated EAE rats
Naive rats
No IgG transfer
EAE Score
3.5
3
2.5
2
1.5
1
0.5
0
6
9
12
15
DAYS

FIG. 7

FIG. 8A

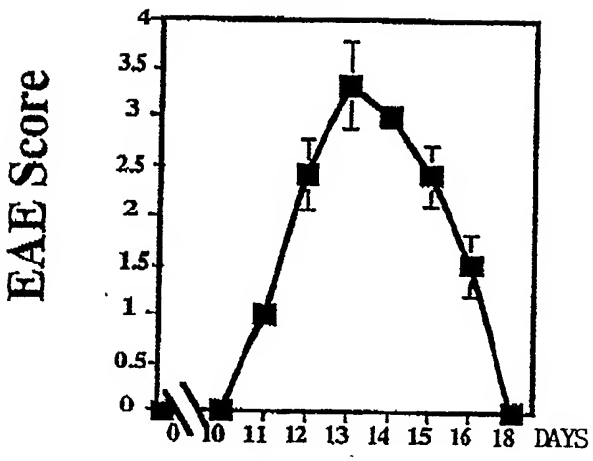


FIG. 8D

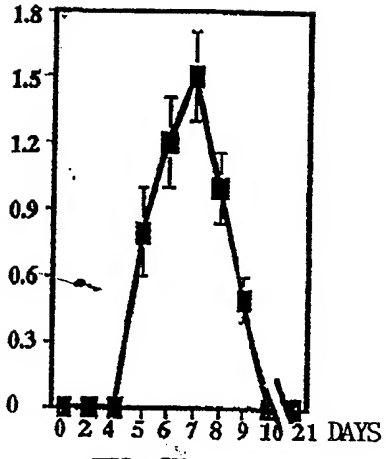


FIG. 8B

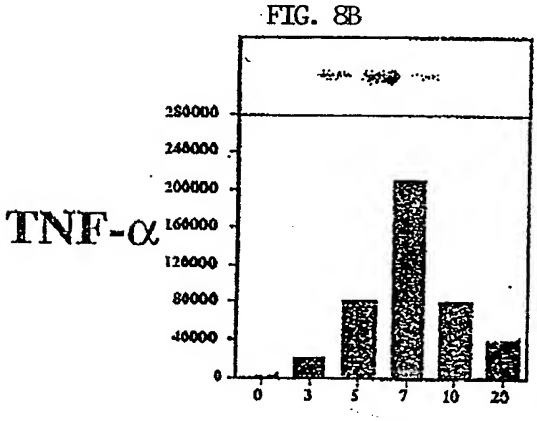


FIG. 8E

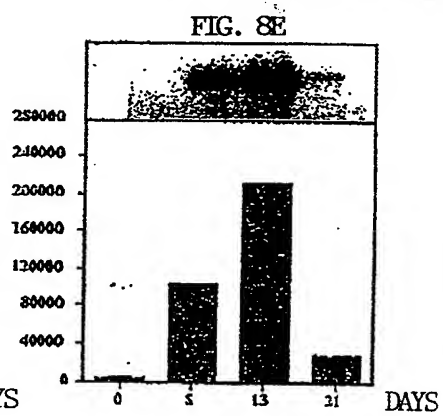


FIG. 8C

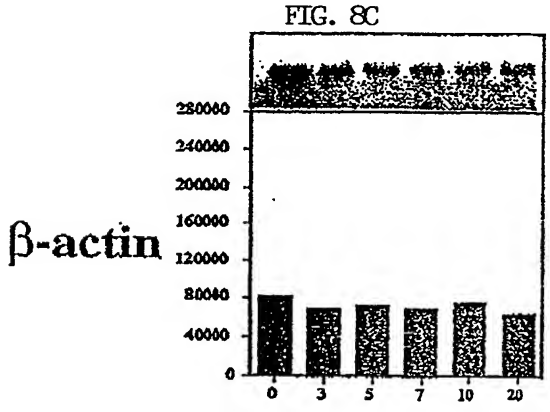
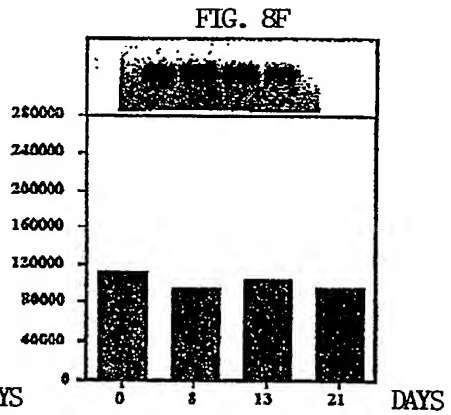


FIG. 8F



Group	DNA vaccination	EAE induction
a	-	-
b	-	CFA alone
c	pcDNA3	-
d	-	+
e	pcDNA3	+
f	pcDNA3-TNF-α	-
g	pcDNA3-TNF-α	CFA alone
h	pcDNA3-TNF-α	+

FIG. 9

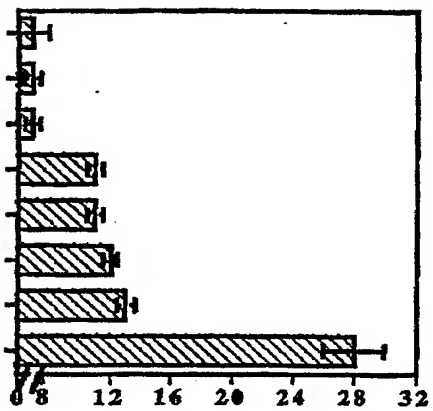


FIG. 10

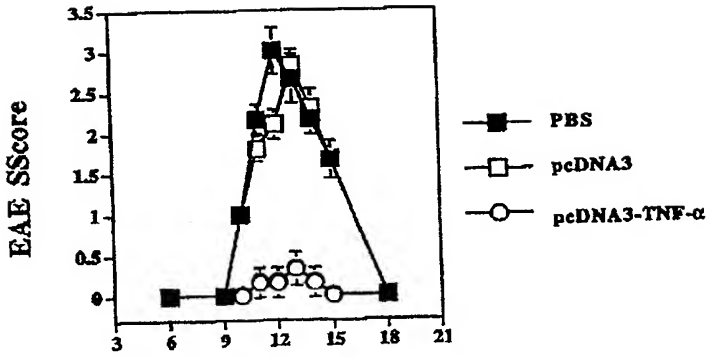


FIG. 11

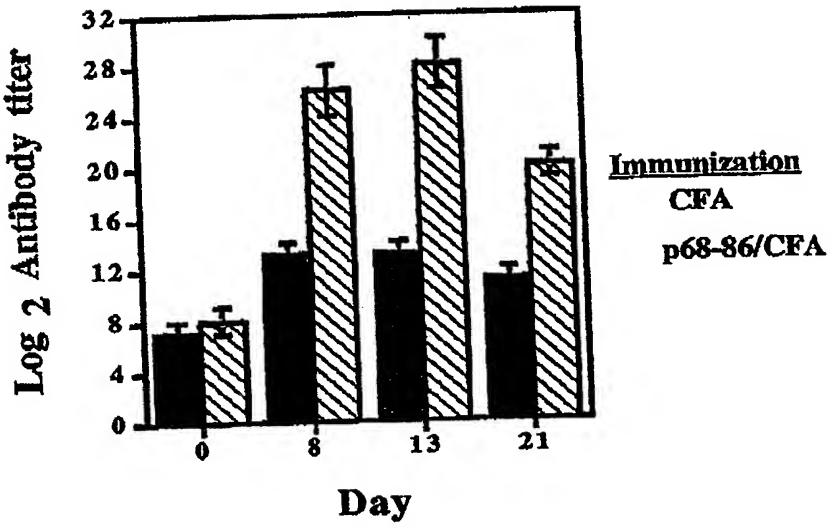
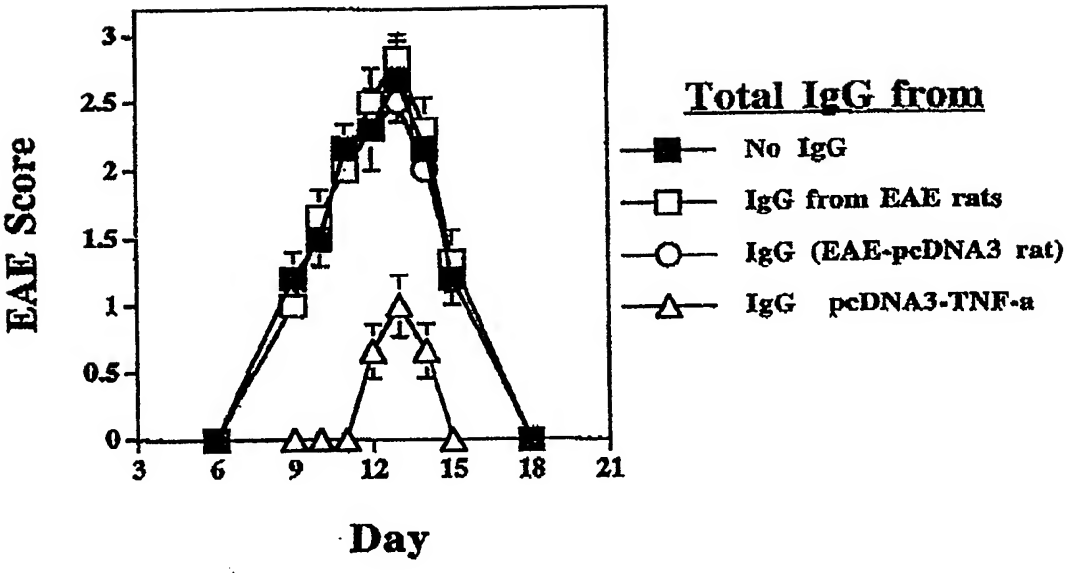

Log 2 Antibody titer
32
28
24
20
16
12
8
4
0
0
8
13
21
Day
Immunization
CFA
p68-86/CFA

FIG. 12


EAE Score
3
2.5
2
1.5
1
0.5
0
3
6
9
12
15
18
21
Day
Total IgG from
No IgG
IgG from EAE rats
IgG (EAE-pcDNA3 rat)
IgG pcDNA3-TNF-a

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Nathan Karin et al.

(ii) TITLE OF INVENTION: DNA VACCINES ENCODING C-C CHEMOKINES AND USE OF SAME FOR PROTECTIVE IMMUNITY AGAINST MULTIPLE SCLEROSIS

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Mark M. Friedman c/o Anthony Castorina

(B) STREET: 20001 Jefferson Davis Highway, Suite 207

(C) CITY: Arlington

(D) STATE: Virginia

(E) COUNTRY: United States of America

(F) ZIP: 22202

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 1.44 megabyte, 3.5" microdisk

(B) COMPUTER: Twinhead® Slimnote-890TX

(C) OPERATING SYSTEM: MS DOS version 6.2, Windows version 3.11

(D) SOFTWARE: Word for Windows version 2.0 converted to an ASCI file

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Friedmam, Mark M.

(B) REGISTRATION NUMBER: 33,883

(C) REFERENCE/DOCKET NUMBER: 910/5

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 972-3-5625553

(B) TELEFAX: 972-3-5625554

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Gly Ser Leu Pro Gln Lys Ser Gln Arg Ser Gln Asp Glu Asn
5 10 15

Pro Val
17

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGAAGGTCT CCACCACTGC CCTTGC 26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCAGGCATTC AGTTCCAGCT CAGTG 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATGAAGCTCT GCGTGTCTGC CTTC 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCAGTTCAAC TCCAAGTCAT TCAC 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGAAGATCT CTGCAGCTGC ATCC 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
CTAGCTCATC TCCAAATAGT TG 22

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
ATGCAGGTCT CTGTCACGCT TCTGGGC 27

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
CTAGTTCTCT GTCATACTGG TCAC 24

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
ATGAGCACAG AAAGCATGAT 20

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
TCACAGAGCA ATGACTCCAA A 21

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATCGTGGGC CGCTCTAGGC A 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGGCCAGCC AAGTCCAGAC G 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/16000

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 15/63; C07K 16/00
US CL :514/44; 424/93.21; 435/320.1; 530/387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/93.21; 435/320.1; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	NAKASHIMA, E. et al. A candidate for cancer gene therapy: MIP-1α gene transfer to an adenocarcinoma cell line reduced tumorigenicity and induced protective immunity in immunocompetent mice. Pharm. Res. 1996, Vol. 13, No. 12, pages 1896-1901, see entire document.	41-51,53 ------ 1-40, 52
X --- Y	NAKASHIMA, E. et al. Synergistic antitumor interaction of human monocyte chemotactant protein-1 gene transfer and modulator for tumor-infiltrating macrophages. Pharm. Res. 1998, Vol. 15, No. 5, pages 685-689, see entire document.	41-51, 53, 54-60 -------- 1-40,52

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
12 OCTOBER 1999

Date of mailing of the international search report
01 NOV 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
ANNE MARIE S. BECKERLEG
JOYCE BRIDGERS
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CHEMICAL MATRIX
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/16000

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SEYA, T. et al. Complement-mediated tumor cell damage induced by antibodies against membrane cofactor protein (MCP, CD46). J. Exp. Med. December 1990, Vol. 172, pages 1673-1680, see entire document.	54-60

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/16000

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WES./BRS, DIALOG: Medline, Biosis, Scisearch, Cancerlit, Embase
search terms: mip, mcp, rantes, ms, multiple sclerosis, plasmid, vector